University of Alberta

Heterologous Expression of Brochocin-C in *Carnobacterium* spp.

by

Valerie M. Bohaychuk ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring, 2004

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-612-96240-7
Our file Notre référence
ISBN: 0-612-96240-7

The author has granted a non-exclusive license allowing the Library and Archives Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou aturement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Abstract

Brochocin-C, a broad spectrum bacteriocin produced by *Brochothrix campestris* ATCC 43754, has the potential to be used as a biopreservative to limit the growth of spoilage and pathogenic bacteria in foods. The wild-type strain of *B. campestris* is not "food-grade" and purification of the bacteriocin is laborious; thus it is necessary to produce brochocin-C in heterologous lactic acid bacteria. The objectives of this research were to develop a stable plasmid vector and to use this vector to produce brochocin-C in *Carnobacterium* spp., and to enhance brochocin-C production by a heterologous host. The genes for brochocin-C production and immunity, including the signal peptide from divergicin A to export the peptide via the general secretory pathway, were inserted into two theta-type replicating plasmids, pTRKH2 and pCD11. Brochocin-C production by *Carnobacterium* spp. was achieved in the absence of antibiotic pressure during incubation in broth media at 25°C. However, only pCD11 was stable in the absence of selective pressure when cultures were incubated at 4°C. The effect of low temperature on the stability of other theta-type plasmids and the ability of the original host bacteria to grow at low temperatures were investigated. The replication region, including 12 and 22 base pair repeats, putative DnaA box and origin of replication, and the *repA* gene of pCD11, were important for plasmid stability during incubation at low temperatures. Insertion of this region into pTRKH2, a plasmid that was not stable at low temperature, increased its stability at 4°C. To enhance brochocin-C production in a heterologous host, several constitutive promoters for chloramphenicol acetyltransferase or brochocin-C expression were screened. Lactococcal promoters, promoters upstream of leucocin A and chloramphenicol acetyltransferase genes, and uncharacterized promoters from *C.*

maltaromaticum were used. Only two out of 19 promoters tested increased brochocin-C production by *Carnobacterium maltaromaticum* LV17C. Real-time polymerase chain reaction showed that the relative gene expression of brochocin-C in *C. maltaromaticum* LV17C was higher when the genes were under the control of the two promoters that increased bacteriocin activity. This research has shown that production of brochocin-C in *Carnobacterium* spp. can be achieved using pCD11 as a stable vector under environmental conditions that simulate food storage and that some constitutive promoters enhance expression.

Acknowledgement

I sincerely thank my supervisor and friend Dr. Lynn McMullen for the guidance and support she has provided throughout my graduate studies. I appreciate the time and effort she has dedicated through the last six years.

I thank Dr. Michael Stiles and Dr. Gordon Greer, members of my supervisory committee, for providing suggestions, encouragement, and support.

I appreciate the friendship of Natisha Rose, Jennifer Snart, Denise Rivard, Susan Gibson, Yan Gao, Rupinder Panayach, Young Nam Kim, and Max Rosario. I especially thank Dr. Charles Franz and Dr. Marco van Belkum for their significant contributions to my research.

I am grateful to Dr. Cornelia Kreplin, John Brown and Ken Manninen at the Food Safety Division, Alberta Agriculture, Food and Rural Development for their support and encouragement. I thank my colleagues in Food Microbiology and I especially thank Barbara Dakin for her friendship and technical assistance. I would also like to acknowledge and thank my colleagues in Molecular Biology for their assistance, guidance, and patience. I am especially thankful to Robin King for her advice, expertise, and friendship.

Finally, I would like to acknowledge my fiancé, Trevor, for his love, support and understanding throughout the years. Also, a special thanks to my family for their constant encouragement and love.

Table of Contents

1. Introduction and literature review........1
  1.1 Lactic acid bacteria and bacteriocins as biopreservatives in food........1
    1.1.1 Food safety and minimally processed foods........1
    1.1.2 Lactic acid bacteria and biopreservation........2
    1.1.3 Bacteriocins and biopreservation........3
  1.2 Bacteriocins produced by lactic acid bacteria........5
    1.2.1 Definition and classification of bacteriocins........5
    1.2.2 Production of class II bacteriocins........7
    1.2.3 Regulation of bacteriocin production........9
  1.3 Transport of bacteriocins from the cell........9
    1.3.1 ABC transporter mechanism........9
    1.3.2 General secretory pathway........11
    1.3.3 Use of transport mechanisms for heterologous expression of bacteriocins...13
  1.4 Heterologous expression systems in lactic acid bacteria........14
    1.4.1 Advantages of heterologous expression........14
    1.4.2 Heterologous expression of bacteriocins using dedicated transport systems........16
    1.4.3 Heterologous expression of bacteriocins using the general secretion pathway........18
  1.5 Plasmids as vectors for heterologous expression........19
    1.5.1 General description of plasmids........19
    1.5.2 Rolling circle replication........19
    1.5.3 Theta-type replication........21
    1.5.4 Factors affecting plasmid stability........23
    1.5.5 Use of plasmids as expression vectors........25
  1.6 Promoters........26
    1.6.1 Promoters and transcription........26
    1.6.2 Promoter activity and strength........27
    1.6.3 Promoters and gene expression........31

1.7 Research objectives.....33
1.8 References.....35
2. Stable expression of brochocin-C by heterologous *Carnobacterium* spp.....46
2.1 Introduction.....46
2.2 Materials and methods.....47
2.2.1 Bacterial strains and culture conditions.....47
2.2.2 DNA isolation and manipulation.....49
2.2.3 Oligonucleotide synthesis and amplification reaction.....50
2.2.4 Construction of vectors for brochocin-C expression.....51
2.2.5 Segregational stability of pVB2-4 and production of brochocin-C.....52
2.2.6 Segregational stability of pCDB and production of brochocin-C.....53
2.2.7 Determination of brochocin-C activity.....53
2.3 Results and discussion.....54
2.3.1 Construction of expression vectors for brochocin-C.....54
2.3.2 Stability of pVB2-4 and pCDB and production of brochocin-C in *Carnobacterium* spp.....55
2.3.3 Brochocin-C activity.....62
2.4 References.....65
3. Effect of low temperature on stability of theta-type plasmids in *Carnobacterium maltaromaticum*.....67
3.1 Introduction.....67
3.2 Materials and methods.....68
3.2.1 Bacterial strains and plasmids.....68
3.2.2 Determination of ability to grow at low temperature.....69
3.2.3 DNA isolation and transformation.....70
3.2.4 Segregational stability.....71
3.2.5 Cloning of replication regions.....71
3.3 Results and discussion.....73
3.4 References.....80
4. Influence of lactic acid bacteria promoters on brochocin-C production by heterologous *Carnobacterium maltaromaticum*.....84

4.1 Introduction..84
4.2 Materials and methods..85
4.2.1 Bacterial strains and plasmids..85
4.2.2 DNA isolation and transformation..85
4.2.3 Oligonucleotide synthesis and amplification reactions..89
4.2.4 Isolation and cloning of LAB promoters..91
4.2.5 Promoter activity in *C. maltaromaticum* LV17C..91
4.2.6 Brochocin-C production in *C. maltaromaticum* LV17C..92
4.3 Results and discussion..93
4.4 References..100
5. Real-time polymerase chain reaction to quantify brochocin-C expression in strains of *Carnobacterium maltaromaticum*..105
5.1 Introduction..105
5.2 Materials and methods..108
5.2.1 Bacterial strains and growth conditions..108
5.2.2 Primer design..110
5.2.3 RNA isolation..110
5.2.4 Reverse transcription..112
5.2.5 Real-time PCR..113
5.2.6 Relative quantification..114
5.3 Results and discussion..115
5.3.1 RNA isolation and reverse transcription..115
5.3.2 Real-time PCR..117
5.4 References..129
6. General discussion and conclusions..134
6.1 References..142

List of Tables

Table 2.1. Bacterial strains and plasmids used in this study.....48

Table 2.2. Production of brochocin-C by *Brochothrix campestris* ATCC 43754 and heterologous *Carnobacterium* spp.....63

Table 3.1. Bacterial strains and plasmids used in this study.....69

Table 4.1. Bacterial strains and plasmids used in this study.....86

Table 4.2. Oligonucleotides used in this study.....90

Table 4.3. Nucleotide sequences of lactococcal and bacteriocin promoter regions.....94

Table 4.4. Chloramphenicol resistance of *C. maltaromaticum* LV17C containing different promoters isolated from lactic acid bacteria and production of brochocin-C determined by the spot-on-lawn assay.....95

Table 5.1. Bacterial strains used in this study and a description of their relevant characteristics.....109

Table 5.2. Relative quantification of brochocin-C gene expression and brochocin-C production by promoter-containing strains of *Carnobacterium maltaromaticum* LV17C.....119

List of Figures

Figure 1.1. Diagrammatic representation of the steps involved in the production of bacteriocins....8

Figure 1.2. ATP binding cassette (ABC) transporter....11

Figure 1.3. Components of the general secretory pathway....12

Figure 1.4. Schematic representation of rolling circle replication....21

Figure 1.5. Theta-type plasmid replication showing unidirectional and bidirectional synthesis....22

Figure 2.1. Segregational stability of pVB2-4 and pTRKH2 in *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26, and *C. divergens* UAL278 grown in APT broth incubated at 25°C....56

Figure 2.2. Growth of *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26, and *C. divergens* UAL278 in APT broth and segregational stability of pVB2-4 during incubation at 4, 6.5, and 11°C....58

Figure 2.3. Growth of *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26, and *C. divergens* UAL278 in APT broth and segregational stability of pTRKH2 during incubation at 4, 6.5 and 11°C....60

Figure 3.1. Plasmid map of pCD11 with relevant restriction sites and locations of primers used in the study....73

Figure 3.2. Growth of bacterial strains in broth media at 4°C....75

Figure 3.3. Stability of plasmids in *C. maltaromaticum* UAL26 grown in APT broth incubated at 25°C and 4°C....76

Figure 3.4. Stability of pTRKH2, pVBL, and pVBS in *C. maltaromaticum* UAL26 grown in APT broth incubated at 4 and 25°C....78

Figure 5.1. Amplification and melting curve analysis of brochocin-C and 16S rRNA gene expression using real-time PCR with three concentrations of $MgCl_2$....121

Figure 5.2. Amplification plots of brochocin-C and 16S rRNA genes....123

Figure 5.3. Standard curves for brochocin-C and 16S rRNA to determine PCR efficiency values....124

Figure 5.4. Amplification plots of brochocin-C and 16S rRNA genes using real-time PCR..........126

List of Abbreviations

A	adenine
ABC	ATP-binding cassette
APT	All Purpose Tween
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AU	arbitrary unit
B.	*Bacillus*
BHI	Brain Heart Infusion
bp	base pair
C.	*Carnobacterium*
C	cytosine
$CaCl_2$	calcium chloride
cDNA	complementary DNA
cfu	colony forming unit
CP	crossing point
CsCl	cesium chloride
C_T	cycle threshold
CTAB	cetyltrimethylammonium bromide
DEPC	diethylpyrocarbonate
DMCS	dimethyldichlorosilane
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded DNA
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
Ent.	*Enterococcus*
G	guanine
g	gram
h	hour(s)

kV	kilovolt
L.	*Lactococcus*
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Leuconostoc*
LB	Luria Bertani
M	molar
min	minute(s)
mg	milligram
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
mRNA	messenger RNA
µg	microgram
µl	microliter
µM	micromolar
ng	nanogram
OD	optical density
Ω	ohm
PCR	polymerase chain reaction
PMF	proton motive force
RNA	ribonucleic acid
RNAP	RNA polymerase
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SEC	general secretion pathway
sec	second(s)
spp.	species
subsp.	subspecies
T	thymine

T.	*Tetragenococcus*
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris hydrochloride
U	unit
UP	upstream
vol.	volume
wt.	weight

1. Introduction and Literature Review

1.1 Lactic acid bacteria and bacteriocins as biopreservatives in food

1.1.1 *Food safety and minimally processed foods*

In recent years food safety has become an increasingly important global concern. It has been estimated that foodborne pathogens cause 76 million illnesses and 5,200 deaths annually in the United States (Mead et al., 1999) and 2.2 million cases occurring each year in Canada (Todd, 1989). As a result, there is enormous economic impact associated with foodborne illness that affects both the food industry and society (Todd, 1989). Media coverage and enhanced knowledge has increased consumer awareness of the way our food is produced, processed and prepared and the importance of food preservation in extending shelf life and reducing potential health hazards. In recent times the food industry has evolved into centralized food processing operations, which has decreased the number of people involved in food production (Ennahar et al., 1999), and international distribution of food products is common. Furthermore, lifestyle changes relating to food preparation and consumption have increased the potential for the mishandling of foods during various stages of processing, storage, distribution, retail, and preparation (Ennahar et al., 1999). In addition, an increased demand for high quality foods that are minimally processed, contain lower levels of preservatives, and that require less preparation time compared with conventional meals has led to the development of refrigerated processed foods with extended storage lives (Peck, 1997). The consumer appeal of these products has resulted in large numbers of minimally processed foods in the retail marketplace. There are several reasons for safety concerns associated with extended storage of minimally processed foods. To maximize the sensory and organoleptic qualities these products are processed at lower temperatures (65 to 95°C) compared with shelf-stable foods (Peck, 1997). The processing temperatures used for production of minimally processed foods reduces the microbial load but does not produce commercial sterility; therefore, adequate refrigeration and other barriers to microbial growth are essential to prevent spoilage and ensure safety. Also, these food products typically have a pH value greater than 5, a high water activity, and a salt concentration of less than 3.5% (Peck, 1997). Thus, these products frequently do not possess any intrinsic inhibitory barriers that will inhibit the growth of pathogens and it is recognized by the scientific community that refrigeration alone can not be relied on to ensure safety (Rhodehamel, 1992). In addition, to extend storage life these food products are frequently packaged under modified atmospheres or vacuum to reduce or eliminate oxygen in the packages. The combination of factors favors the growth of heat resistant, psychrotrophic, foodborne pathogens that can grow in environments with little or no oxygen. One of the strategies being investigated to increase food safety and quality is the use of antimicrobial peptides from lactic acid bacteria (LAB) that target pathogens and spoilage bacteria without toxic or other adverse effects and these LAB are being comprehensively studied for potential food applications (Cleveland et al., 2001).

1.1.2 *Lactic acid bacteria and biopreservation*

Recently, several large outbreaks of foodborne illness and recalls of millions of kilograms of food have highlighted the need for additional methods to increase food safety. It has been suggested that in addition to the primary barrier of adequate refrigeration there should be multiple barriers or hurdles incorporated into perishable foods with extended storage lives to ensure safety (Rhodehamel, 1992). In the past, artificial chemical preservatives have been employed to limit the number of microorganisms capable of growing in food, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced by LAB as biopreservatives (Abee et al., 1995). Biopreservation technology using LAB and/or their bacteriocins could be a very desirable means of introducing an additional hurdle to the growth of pathogens and spoilage bacteria in minimally processed food products.

Biopreservation refers to extended storage life and enhanced safety of foods using natural microflora and their antibacterial products (Ross et al., 2002; Stiles, 1996). Biopreservatives can be used in a number of ways in food systems, from the addition of concentrated bacteriocin preparations as food additives to the use of the bacteriocin-producing bacterial strains directly in foods as starter or protective cultures (Ross et al., 2002). Several LAB have potential for use as biopreservatives because they are safe to consume and during storage they naturally dominate the microflora of many foods (Stiles, 1996). Also, almost all the different species which make up the LAB group have been reported to produce bacteriocins (Ross et al., 2002). There are many strains of LAB that will grow in refrigerated foods; however, not all of these are ideal for use as biopreservatives. Storage life of food can be shortened if the wrong type of LAB are present, for example if hydrogen sulfide-producing *Lactobacillus sakei* strains predominate (Stiles, 1994) and produce offensive off odors. Metabolic end products such as organic acids, hydrogen peroxide, and carbon dioxide are produced by some LAB in amounts that could cause spoilage of the food products. If LAB are to be used as biopreservatives for controlling the growth of pathogens and extending storage life, the impact of these organisms on the sensory properties of the product must be carefully considered (McMullen and Stiles, 1996).

One of the genera of LAB that has promising potential for use as a biopreservative is *Carnobacterium*. *Carnobacterium* spp. were described in the past as nonaciduric lactobacilli and are commonly associated with refrigerated meat, poultry, and fish (Hammes et al., 1992; Klaenhammer, 1993; Stiles and Holzapfel, 1997). *Carnobacterium* spp. are heterofermentative but produce only small volumes of carbon dioxide (Collins et al., 1987; Mora et al., 2003) and produce less acid than some other LAB. Also, they do not produce hydrogen sulfide (Hammes et al., 1992). Carnobacteria are unlikely to cause spoilage of food products because of these factors.

1.1.3 *Bacteriocins and biopreservation*

Lactic acid bacteria (LAB) can be useful in the preservation of foods because of the inhibitory effects of the metabolic end products such as lactic acid on other bacteria; however, production of bacteriocins would enhance their biopreservative potential. Bacteriocins produced by LAB, such as nisin, pediocin, and leucocin, have been shown to reduce bacterial numbers in food systems (Delves-Broughton et al., 1996; Leisner et al., 1996; Luchansky et al., 1992; Muriana, 1996). The application of bacteriocins from LAB in combination with traditional methods of preservation and proper hygienic processing could be effective in controlling spoilage and pathogenic bacteria; however, a number of problems such as low production levels, instability in certain environments, inactivation, and the development of resistance in target bacteria have been encountered (Abee et al., 1995; Ennahar et al., 1999). The inactivation of a bacteriocin in a food product was recently documented. Nisin, a broad spectrum bacteriocin has been licensed for use as a food preservative in over 50 countries (Delves-Broughton et al., 1996), but is ineffective in raw meat products. Glutathione S-transferase, an enzyme found in fresh meats, catalyzes a reaction between nisin and glutathione, thereby inactivating the bacteriocin (Rose *et al.*, 1999; Rose *et al.*, 2002). The interaction of food components with bacteriocins is an area that needs additional research if the use of bacteriocins as biopreservatives is to become a viable option. The resistance of target organisms to various bacteriocins is, unfortunately, a common phenomenon. In a recent study, a large number of strains of *Listeria* were investigated and a few strains (5 out of 31) were found to be naturally resistant to high concentrations of pediocin AcH, divercin V41, enterocin A, and mesentericin Y105 (Ennahar et al., 2000a). Various strains of *Listeria monocytogenes* have shown varying degrees of acquired resistance after exposure to leucocin A (Vadyvaloo et al., 2002), pediocin PA-1, nisin A (Gravesen et al., 2002; Rasch and Knochel, 1998), leucocins A, B and E, sakacin A (Dykes and Hastings, 1998), bavaricin A (Rasch and Knochel, 1998), or divercin V41 (Duffes et al., 2000). These limitations can seriously impact the usefulness of bacteriocins in food systems.

A recent trend in bacteriocin research, the heterologous expression of bacteriocins among various LAB strains, offers an excellent tool that may help overcome these obstacles (Ennahar et al., 1999). Recombinant DNA technology is being applied to enhance production, to transfer bacteriocin genes to other species, and for mutation and selection of bacteriocin variants with increased and/or broader activity spectra (Abee et al., 1995). In addition, heterologous expression can be used to develop LAB that produce multiple bacteriocins, each one having its own specific range of target bacteria. This could greatly enhance the antimicrobial efficiency of LAB in food by yielding bacteriocin-producing strains that inhibit the growth of a broad range of undesirable organisms and possibly reducing the risk of bacteriocin-resistance developing among the target bacteria (Ennahar et al., 1999). The tools of genetic engineering have enormous potential for increasing the effectiveness of bacteriocin-producing LAB as biopreservatives to enhance the safety and storage life of minimally processed foods.

1.2 Bacteriocins produced by lactic acid bacteria

1.2.1 *Definition and classification of bacteriocins*

Lactic acid bacteria (LAB) have a long history of use in the preservation of foods through the production of metabolic end products and antimicrobial substances known as bacteriocins. Bacteriocins are a heterogeneous group of ribosomally synthesized, antibacterial proteins or peptides that vary in spectrum of activity, mode of action, molecular weight, genetic origin, and biochemical properties (Abee et al., 1995). Bacteriocins have been extensively studied in recent years and during the past decade the DNA sequences of a large number of bacteriocin genetic loci have been determined (Eijsink et al., 2002). Through intensive investigations several interesting aspects of bacteriocins have been revealed such as regulation of production, structure-function relationships, as well as aspects of immunity, target cell sensitivity, and resistance (Eijsink et al., 2002). Bacteriocins have been grouped into different classes by researchers using various criteria. In one of the earlier publications, four classes of bacteriocins were defined based on knowledge of structure and mechanism of action (Klaenhammer, 1993). Class I contained the lantibiotics, which are small membrane-active peptides containing unusual amino acids (lanthionine, β-methyllanthione) and dehydrated residues. Class II was composed of small, heat stable, non-lanthionine containing membrane-active peptides and was separated into three subclasses. Class IIa contained *Listeria*-active peptides with a consensus sequence in the N-terminal of YGNGVXC. Class IIb consisted of poration complexes with two or more proteinaceous peptides needed for activity. Thiol-activated peptides requiring reduced cysteine residues for activity comprised class IIc. Class III was composed of large, heat-labile proteins and Class IV contained complex bacteriocins composed of proteins with one or more chemical moieties such as lipids or carbohydrates that were needed for activity. Subsequently it was determined that direct evidence for the necessity of these additional chemical moieties was lacking and it was suggested that the complexes could in fact be artifacts caused by interaction of the proteins with cell or medium constituents (Nes et al., 1996). Nes et al. (1996) discussed the biosynthesis of bacteriocins in LAB and divided bacteriocins into three classes. Class I: the lantibiotics; class II: the small heat stable non-lantibiotics; and class III: the large heat labile bacteriocins. The class II bacteriocins were further divided into three subclasses: IIa: pediocin-like bacteriocins with strong antilisterial effect; IIb: two-peptide bacteriocins; and IIc: *sec*-dependent secreted bacteriocins. In their subsequent review, (Nes and Holo, 2000) maintained the three main classes but subdivided the lantibiotics (class I) into two types: Type A, elongated molecules and Type B, globular molecules and also redefined class IIc as 'other bacteriocins' instead of *sec*-dependent secreted bacteriocins.

In recent years several more class II bacteriocins from LAB have been identified and characterized. Previously, classification was largely based on the presence of a YGNGVXC amino acid motif near the N-terminus of the active peptide, the presence of cysteine residues resulting in disulfide bridges, and *sec*-dependent bacteriocins were classified as a separate subgroup (van Belkum and Stiles, 2000). Van Belkum and Stiles (2000) proposed the following classification scheme for class II nonlantibiotic antibacterial peptides. Class IIa contained peptides called cystibiotics with two disulfide bridges resulting from four cysteine residues, one in the N-terminal half the other in the C-terminal half of the molecule; class IIb consisted of cystibiotics with one disulfide bridge resulting from two cysteine residues in the N-section of the peptide and is the largest group; class IIc was comprised of cystibiotics with one disulfide bridge that spans the N- and C- sections of the peptide but lacks the YGNGVXC motif of class IIa and IIb bacteriocins. Class IId were peptides containing one or no cysteine residues and do not contain the YGNGVXC motif, class IIe were two-peptide bacteriocins that are either type E (enhancing) or type S (synergistic), and class IIf contained atypical bacteriocins such as cyclic peptides and leaderless peptides. Regardless of the scheme used to classify bacteriocins, it is evident that the number of well-characterized bacteriocins has increased dramatically in recent years and that we have more knowledge of the genetics, structure and function of bacteriocins.

1.2.2 *Production of class II bacteriocins*

The majority of bacteriocin research is currently being conducted on the small, heat stable, non-lanthionine-containing peptides (class II bacteriocins), especially in regards to their potential use as biopreservatives in foods. The majority of class II bacteriocins require a minimum of four genes for production, immunity, and export. These genes encode the prebacteriocin, the immunity protein, the ATP-binding cassette (ABC) transport protein, and the accessory protein. Bacteriocins that are regulated require an additional three genes that encode for the induction peptide, histidine protein kinase, and the response regulator (Figure 1.1 D). The steps involved in the production of class II bacteriocins include: i) synthesis of prebacteriocins and/or induction factor prepeptides, as well as the proteins for immunity, export, and regulation; ii) processing and export of the mature bacteriocin and/or induction peptides using the ABC transporter and accessory proteins; iii) immunity; and iv) regulation of bacteriocin production involving induction peptides, histidine protein kinase and response regulators (Figure 1.1).

Bacteriocins are synthesized as prebacteriocins (Figure 1.1 A) that contain an N-terminal extension, encoded by a leader sequence, which is cleaved during export to form the mature bacteriocin (Figure 1.1 B). The leader sequence has the function of preventing the bacteriocin from being biologically active while inside the producer cell and to serve as a recognition signal for the ABC transporter (Nes et al., 1996). To protect a bacteriocin-producing cell from being killed by its own bacteriocin, each bacteriocin also concurrently expresses its own dedicated protein that confers immunity (Figure 1.1 C). The immunity protein is functionally expressed in the absence of processing and export machinery even though the synthesis of an extracellular bacteriocin requires transport mechanisms (Holo et al., 1991). It has also been found that LAB may contain

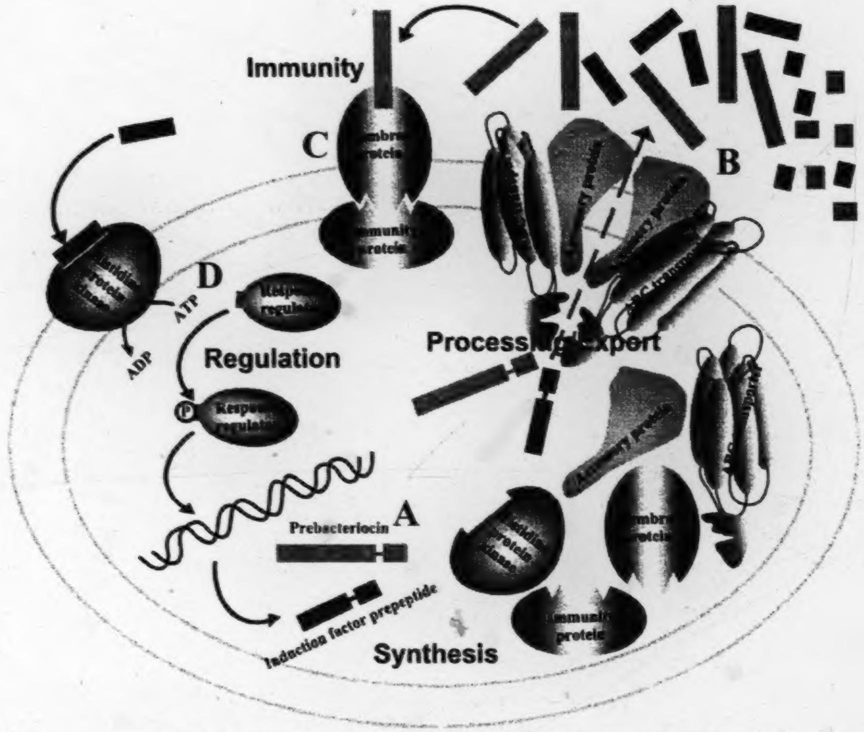

Figure 1.1. Diagrammatic representation of the steps involved in the production of bacteriocins. Figure based on Ennahar et al. (2000b) and Nes et al. (1996).

varying numbers of immunity genes that provide variable degrees of protection towards a variety of subgroups of bacteriocins and may also differ in terms of expression levels (Eijsink et al., 1998; Eijsink et al., 2002; Franz et al., 2000). In addition to the genes for the bacteriocin prepeptide and immunity proteins, there are also genes for the ABC transporter and accessory protein, which will be described in more detail later. A few characterized bacteriocins are secreted from the cell by the translocase general secretion (Sec) pathway (Cintas et al., 1997; Tomita et al., 1996; Worobo et al., 1995). These bacteriocins are also produced as prepeptides but with an N-terminal signal peptide rather than a leader peptide. The genes for the dedicated ABC transporter and accessory protein are absent from these bacteriocins' operons. The details of secretion of peptides via the Sec pathway will be discussed later.

1.2.3 *Regulation of bacteriocin production*

Occasionally bacteriocinogenic LAB loose the ability to produce bacteriocins. Loss of bacteriocin production could be due to plasmid loss or to transposition-mediated inactivation but it has been shown that production of some bacteriocins is transcriptionally regulated and these bacteriocins require an induction factor for production (Axelsson and Holck, 1995; Brurberg et al., 1997; Diep et al., 1996; Franz et al., 2000; Huhne et al., 1996; Nilsen et al., 1998; Quadri et al., 1997). Nucleotide sequencing of Class II bacteriocins' operons has revealed that regulatory systems are composed of genes encoding a secreted induction factor and proteins that are homologous to histidine kinases and response regulators (Axelsson and Holck, 1995; Diep et al., 1996; Franz et al., 2000; Huhne et al., 1996). Regulated bacteriocins frequently contain regulatory-like boxes with conserved direct repeats upstream of the –35 sites of the promoter regions whereas the nucleotide sequences of constitutively produced bacteriocins do not contain these direct repeats (Nes et al., 1996). Nes et al. (1996) indicated that these regulatory-like boxes found in the promoters likely serve as binding sites for the transcription regulators and that the transcription regulators are most probably response regulators. For use in food systems it may be more desirable to have bacteriocins produced constitutively than bacteriocins produced using regulated promoters because induction factors are not necessary. Inducible systems are not always easy to manage under industrial conditions and a constitutive promoter with a desired level of expression may be more efficient (Renault, 2002).

1.3 Transport of bacteriocins from the cell

1.3.1 *ABC transporter mechanism*

The bacteriocin structural gene encodes a precursor peptide that is processed and secreted by either dedicated machinery belonging to the ATP-binding cassette (ABC) superfamily or via the Sec-dependent pathway. Most of the research conducted on the transport of peptides/proteins in bacteria has been done using *Escherichia coli* as a model system. *E. coli* is a good model for transport systems in bacteria and most of the information can be applied to both gram positive and gram negative bacteria. Bacteriocins that contain double-glycine-type leader peptides use a dedicated transport system consisting of two different membrane-bound proteins, the ABC transporter and the accessory protein (Figure 1.1 B). The bacteriocin ABC transporter gene is usually either part of the bacteriocin operon or found on a separate operon in the vicinity of the bacteriocin-containing operon (Diep et al., 1996; Franz et al., 2000; Fremaux et al., 1995; McCormick et al., 1998; van Belkum and Stiles, 1995). ABC transporters utilize the energy of adenosine triphosphate (ATP) hydrolysis to pump substrate across the membrane and each ABC transporter is relatively specific for a given substrate (Higgins, 1992). ABC transporters require the function of multiple polypeptide/protein domains organized in a characteristic fashion that typically consists of four membrane-associated domains (Figure 1.2).

The transmembrane domains are highly hydrophobic and each domain normally consists of six membrane-spanning α-helical segments [Figure 1.2 A1, A2; (Bolhuis et al., 1997; Franke et al., 1999; Higgins, 1992)]. These transmembrane domains form the pathway through which substrates cross the membrane (Higgins, 1992). The nucleotide binding domains (Figure 1.2 B1, B2) are hydrophilic and are peripherally located at the cytoplasmic face of the membrane. The nucleotide binding domains contain the highly conserved ATP-binding cassette which binds ATP and couples ATP hydrolysis to the transport process (Bolhuis et al., 1997; Higgins, 1992). It is thought that the nucleotide binding domain of the ABC transporter binds to the prepeptide leader sequence, which triggers ATP hydrolysis and subsequent conformational changes in the transporter, resulting in the leader peptide's removal and translocation of the mature bacteriocin across the cytoplasmic membrane (Ennahar et al., 2000b). The nucleotide binding domain is located in the cytoplasm thus processing of the precursor into the mature form takes place at the cytosolic side of the cytoplasmic membrane (Young and Holland, 1999).

In addition to the ABC transporter, a second component that is essential for bacteriocin transport is the accessory protein. Although it is postulated that the accessory proteins facilitate membrane translocation and/or help in the processing of the leader peptide, their specific role is still not understood (Ennahar et al., 2000b; Franke et al., 1999; van Belkum and Stiles, 2000).

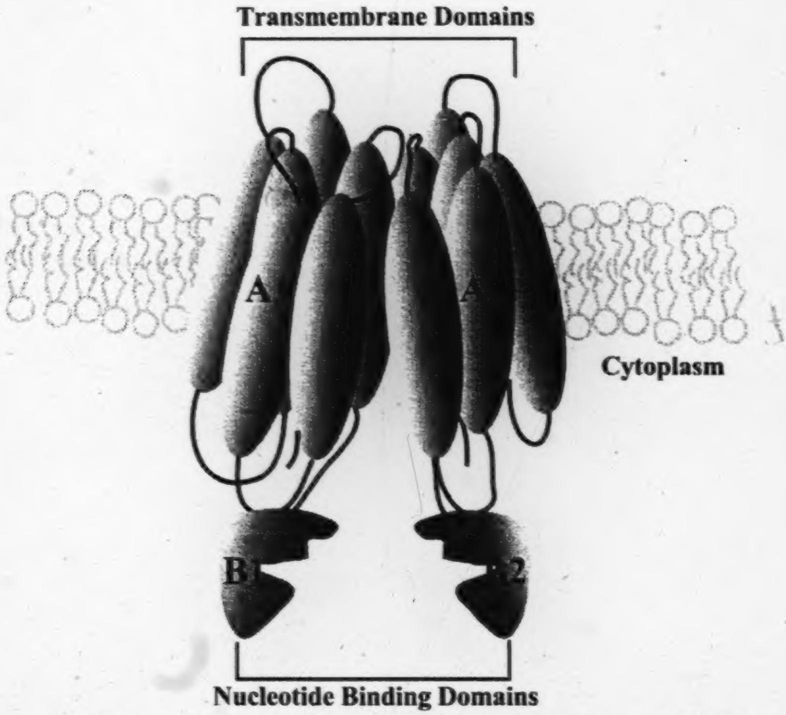

Figure 1.2. ABC transporter consisting of two transmembrane domains that are present in the phospholipid bilayer and two nucleotide binding domains that are located on the cytoplasmic side of the membrane. Figure adapted from Bolhuis et al. (1997).

1.3.2 *General secretory pathway.*

A few bacteriocins contain a signal peptide rather than a leader peptide and are exported out of the cell via the general secretion (Sec) pathway. The Sec pathway is generally responsible for the transport of newly synthesized proteins out of the cytoplasm before they acquire their final structures and in bacteria the Sec system is essential for cell viability (Economou, 1999; Mori and Ito, 2001). The Sec pathway is used by hundreds of substrates that do not share any sequence similarity but the information on the final cellular destination of all secretory polypeptides is contained in the signal peptide, a 20 to 30 residue amino-terminal extension (Economou, 1999). Signal peptides mediate the initial recognition of immature polypeptides by the cell and are proteolytically removed on the *trans* side of the membrane (Economou, 1998). The Sec pathway is divided into three distinct but sequential and interdependent stages: Stage I, targeting; Stage II, translocation; and Stage III, release (Figure 1.3) (Economou, 1999).

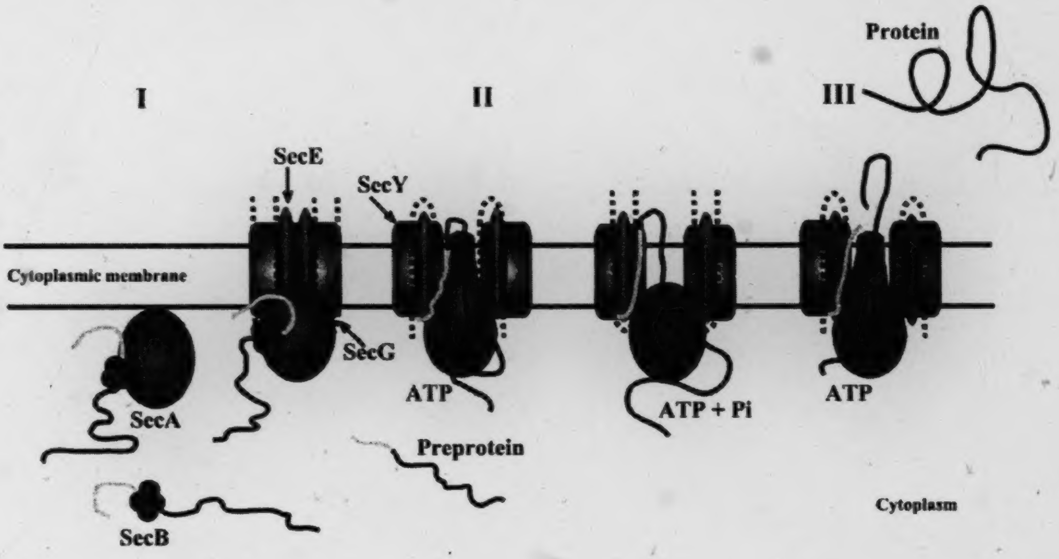

Figure 1.3. Components of the general secretory pathway with the three main stages involved in export.
(The preprotein is represented by the black line with the grey region showing the signal peptide. Figure based on Mori and Ito (2001) and Economou (1999). See text for detailed explanation).

During Stage I, the preprotein substrates are targeted when the signal peptide is recognized by the Sec machinery and the preproteins are then guided to exit sites in the membrane by association with chaperones such as SecB (Figure 1.3 I). The signal peptide plays an important role by delaying the folding of the whole polypeptide thereby allowing the chaperones to bind to the preprotein. The binding of chaperones to the preproteins further prevents folding and aggregation (Economou, 1998). The preprotein and chaperone complexes, which have a high affinity for the translocase, bind to the membrane receptor, SecA, which is the peripheral subunit of the translocase. The translocase is also comprised of the integral membrane polypeptides, SecY, SecE, and SecG (Figure 1.3) (Economou, 1998). Translocation occurs during Stage II where the preprotein is moved across the membrane (Figure 1.3 II). SecA binding is facilitated by the signal peptide and uses the energy from ATP to fuel cycles of membrane insertion and de-insertion (Economou, 1998). One of the notable features of the bacterial preprotein translocase is that it uses both ATP and proton-motive force (PMF) energy sources. The two energy components act at distinct stages of the translocation reaction, with SecA, an ATPase (Oliver, 1993), utilizing ATP first (Schiebel et al., 1991). The PMF is always present but its contribution is seen after the preprotein has translocated about halfway through the membrane and the PMF can drive completion of translocation in the absence of ATP if SecA is inactivated (Schiebel et al., 1991). ATP hydrolysis by SecA leads to a change in the proteolytic accessability of the preprotein (Economou, 1998) allowing the release of the substrate from SecA (Schiebel et al., 1991). The preproteins are threaded through the translocase, the signal peptide is cleaved by the signal peptidase, and the remaining part of the mature peptide is no longer attached to the membrane and can begin folding (Figure 1.3 III). The mature peptide is then secreted from the cell.

1.3.3 *Use of transport mechanisms for heterologous expression of bacteriocins.*

Knowledge of the transport mechanisms employed by bacterial cells has been useful in the investigation of heterologous expression of bacteriocins. Utilization of the significant amino acid homology in the leader peptides and the dedicated transporters of most class II bacteriocins is an appealing option for the heterologous production of bacteriocins (Rodriguez et al., 2003) because various bacteriocins could be exported using the dedicated transport mechanisms of heterologous bacteria. The effectiveness of this strategy was fittingly demonstrated with three class II bacteriocins, leucocin A, lactococcin A, and colicin V (van Belkum et al., 1997). The leader peptides of leucocin A, lactococcin A or colicin V were fused to divergicin A, a bacteriocin from *Carnobacterium divergens* that is secreted via the cell's Sec pathway (van Belkum et al., 1997). The researchers showed that leucocin A, lactococcin A, and colicin V leader peptides can direct the secretion of divergicin using the leucocin A, lactococcin A as well as the colicin V secretion apparatus in *Leuconostoc gelidum, Lactococccus lactis,* and *E. coli*, respectively. The leader peptide of leucocin A can direct the secretion of divergicin A in *Lc. gelidum, L. lactis* and *E. coli* if the transport proteins for leucocin A, lactococcin A and colicin V, respectively, are present. In addition, the leucocin A leader peptide is able to direct the secretion of colicin V in *L. lactis* when the genes for the transport proteins for lactococcin A are included (van Belkum et al., 1997). This strategy is clearly a viable option for the heterologous production of bacteriocins by LAB.

Production of bacteriocins in heterologous hosts using a different approach, the in-frame fusion between sequences encoding the Sec pathway signal peptide together with the mature part of the bacteriocin has been demonstrated. Fusing the signal peptide from divergicin A in place of the leader peptide of carnobacteriocin B2 allowed the export of the active bacteriocin in the absence of its dedicated machinery in heterologous *Carnobacterium* spp. (McCormick et al., 1996). A similar strategy was used to achieve the secretion of separate components of the two-peptide bacteriocin, brochocin-C, via the Sec pathway by heterologous *Carnobacterium* spp. by fusing the signal peptide of divergicin A in the place of each of the leader peptides (McCormick et al., 1998). The heterologous expression of bacteriocins utilizing either the ABC or Sec transport mechanisms will be discussed in more detail.

1.4 Heterologous expression systems in lactic acid bacteria.

1.4.1 *Advantages of heterologous expression.*

Heterologous expression may offer an attractive means of overcoming some of the challenges that could arise from the production of bacteriocins by LAB when used as biopreservatives. The effectiveness of bacteriocins or bacteriocin-producing strains in foods can be limited by factors such as a narrow activity spectra, spontaneous loss of bacteriocinogenicity, poor adaptation of the natural host to food environments, deleterious sensorial effects caused by the growth of the natural host in foods, and the emergence of bacteriocin-resistant spoilage or pathogenic bacteria (Rodriguez et al., 2003). Use of recombinant technology has the potential to confer antimicrobial properties to strains of technological interest such as those used as starter cultures and for construction of multibacteriocinogenic strains (Rodriguez et al., 2003). Expression systems are typically comprised of host cells and genetic elements, such as promoters, regulatory factors if necessary, genes of interest, and plasmids (at varying copy numbers and stabilities) as vectors, and the choice of each component can be critical for the successful expression of the gene of interest (Billman-Jacobe, 1996; Rodriguez et al., 2003). *E. coli* has long been considered the primary prokaryotic host for cloning and expressing heterologous genes due to its extensive genetic characterization (Rodriguez et al., 2003). However, a single organism is unlikely to be suitable for every application and not every gene can be expressed efficiently in *E. coli* (Makrides, 1996; Rodriguez et al., 2003). This may be due to the unique and subtle structural features of the gene sequence, the stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by the host cell proteases, major differences in codon usage between the foreign gene and native *E. coli* (many LAB have a G+C content of about 35% compared to 50% for *E. coli*), and the potential toxicity of the protein to the host (Makrides, 1996). The toxicity of a heterologously expressed bacteriocin to the host was demonstrated by McCormick et al. (1998). The signal peptide of divergicin A was fused in the place of the leader peptides of the two-component bacteriocin, brochocin-C. The bacteriocin was produced by *Carnobacterium* spp. using the Sec pathway as an export system; however, the researchers failed to generate an expression plasmid in *E. coli* and concluded that this was probably a result of the toxicity of the bacteriocin to the cell (McCormick et al., 1998). In addition, *E. coli* do not have generally-recognized-as-safe (GRAS) status and can be potential foodborne pathogens. Many LAB are food-grade, which are organisms that have a history of safe use in foods, making them potentially useful for the heterologous production of commercially important proteins or peptides (Rodriguez et al., 2003).

1.4.2 *Heterologous expression of bacteriocins using dedicated transport systems.*

The secretion and processing of most class II bacteriocins requires a dedicated transport mechanism comprised of an ABC transporter and an accessory protein. Antimicrobial peptides may be more efficiently exported using these dedicated systems compared with use of general secretion mechanisms (Rodriguez et al., 2003). Many bacteriocins have been successfully produced by heterologous LAB using the dedicated transport system. A system based on introducing two plasmids into a bacteriocin-negative *Lactobacillus sakei* strain was developed (Axelsson et al., 1998). One plasmid contained the genes necessary for transcriptional activation of the sakacin A promoter, and the genes encoding the proteins needed for export and processing. The second plasmid contained the structural and immunity genes of sakacin P, pediocin PA-1, or piscicolin 61 fused to the sakacin A promoter. The level of production of these bacteriocins by *L. sakei* was in the same range or higher as those obtained from the wild-type producer strains. This study confirmed the general picture that double-glycine-type leader peptides tend to be recognized by transport machineries for unrelated bacteriocins (Axelsson et al., 1998).

The dedicated transport machinery of *Lactococcus lactis* IL1403 was used for the heterologous expression of pediocin PA-1 (Horn et al., 1998). In this study a vector containing an in-frame fusion between sequences encoding the lactococcin A leader and the structural part of mature pediocin PA-1 was constructed and the hybrid genes were introduced into *L. lactis* (Horn et al., 1998). Although pediocin PA-1 was produced by *L. lactis,* the level of production was approximately 25% of that produced by the parental strain, *Pediococcus acidilactici* (Horn et al., 1998). The researchers speculated that the reduced production may have been due to the low copy number of the chromosomal *lcnC* and *lcnD* gene analogues whose products form the transport system for the translocation of lactococcin. This could have resulted in less efficient secretion of the bacteriocin or the products of these genes were not identical to the equivalent lactococcin A translocatory apparatus (Horn et al., 1998). In a subsequent study, the *lcnC* and *lcnD* genes from a lactococcin A-producing strain were introduced into the pediocin PA-1-producing *L. lactis* IL1403, which resulted in production levels that were greater than 95% that of the natural pediocin producer (Horn et al., 1999). Also, a nisin A-producing strain, *L. lactis* subsp. *cremoris* FI5876 was transformed with the recombinant plasmids used for the heterologous production of pediocin PA-1 with the resulting production of both bacteriocins. However, pediocin PA-1 production was only 11% of that produced by the parental strain while nisin A production was 96% of that produced by the parental strain (Horn et al., 1999). Other bacteriocins such as lactacin F, which is composed of LafA and LafX peptides and is produced by *Lactobacillus johnsonii*, was heterologously produced by *Carnobacterium maltaromaticum* LV17, a carnobacteriocin-producing strain (Allison et al., 1995). This study demonstrated that even without the replacement of the leader peptides, the processing and export machinery of the carnobacteriocins could be used for the heterologous production of lactacin F peptides. However, the lactacin F peptides were expressed at a lower level than the carnobacteriocins likely as a result of the bacteriocin precursors not competing efficiently for the native processing and secretion recognition signals (Allison et al., 1995). These studies demonstrated that one of the limitations of heterologous production of bacteriocins is a reduced level of production compared to the native producer strains.

To demonstrate the advantage of utilizing the dedicated transport system over the Sec pathway for the heterologous expression of bacteriocins, Biet et al. (1998) compared the heterologous expression of mesentericin Y105 using either the dedicated transport system or the general secretory pathway. Mesentericin Y105 is a class II bacteriocin produced by *Leuconostoc mesenteroides* Y105 and is exported with a dedicated transport system. The signal peptide of divergicin A was fused in front of the mature form of mesentericin Y105 and its immunity protein, and the secretion vector, FBYC07, was transformed into *Lc. mesenteroides* DSM 20484 and mesentericin Y105 was heterologously expressed via the Sec pathway. To compare the two transport systems, a vector, FBYC04, that contained the mesentericin Y105 structural gene, its immunity gene, and the genes that encode the two components of a putative ABC transport system was transformed into *Lc. mesenteroides* DSM 20484 and heterologous expression was achieved. The bacteriocin was purified and the amount of mesentericin Y105 produced by *Lc. mesenteroides* DSM 20484(pFBYC04) was similar to that produced by the wild-type strain, *Lc. mesenteroides* Y105 (142 and 129 μg 100 ml^{-1}, respectively), whereas only 34 μg 100 ml^{-1} was obtained from *Lc. mesenteroides* DSM 20484(pFBYC07). Although the yield of mesentericin Y105 was much lower using the Sec pathway, the authors did concede that the reduced level may not only be related to the efficiency of the export system itself but also to gene copy number since different plasmid vectors were used for each system and/or promoter efficiencies since different promoters were also used (Biet et al., 1998). Despite certain limitations, use of the dedicated transport machinery offers an attractive approach for the heterologous production of bacteriocins.

1.4.3 *Heterologous expression of bacteriocins using the general secretion pathway.*

Although the production of some bacteriocins may be greater using the dedicated transport mechanisms, the heterologous production of bacteriocins via the general secretory (Sec) pathway may still be a valid strategy in some cases. Manipulation of bacteriocin genes using the Sec pathway has an advantage in that it requires much less genetic material than the use of the dedicated transport system because there is no need to include the genes encoding the ABC transporter and accessory protein. Several researchers have demonstrated the usefulness of the Sec pathway for the heterologous expression of bacteriocins in LAB. Enterocin B (Franz et al., 1999), brochocin-C (McCormick et al., 1998), carnobacteriocin B2 (McCormick et al., 1996), mesentericin Y105 (Biet *et al.*, 1998; Biet *et al.*, 2002) and colicin V (McCormick et al., 1999) were heterologously expressed in LAB using the Sec pathway by fusing the signal peptide from divergicin A in place of the double glycine-type leader peptides. Export of carnobacteriocin B2 was shown to be less efficient (100 units of activity per ml) using the Sec pathway in comparison with production from the wild-type organism (400 units of activity per ml) (McCormick et al., 1996). Likewise, the heterologous production of mesentericin Y105 using the Sec pathway was approximately 3 times less (~300 μg l^{-1}) compared to production by the natural producer (>1 mg l^{-1}) (Biet et al., 1998). However, when a different plasmid vector, a derivative of pTXL1, was used, mesentericin Y105 was produced at a level similar to that of the natural producer (Biet et al., 2002). This suggests that there is potential, if the appropriate plasmids and promoters are used, to use the Sec pathway to produce bacteriocins in heterologous LAB at the same or similar concentrations that are produced by the wild-type organisms.

1.5 Plasmids as vectors for heterologous expression.

1.5.1 *General description of plasmids.*

Researchers have extensively used plasmids as vectors for bacteriocin genes. This has allowed for the heterologous expression of bacteriocins by LAB through relatively simple genetic manipulations. Plasmids are extrachromosomal DNA elements with characteristic copy numbers within the host, they replicate in an autonomous and self-controlled way, and may constitute a substantial amount of the total genetic content of an organism (del Solar et al., 1998). There are three main factors that have contributed to the development of plasmid research: the genetic organization of these elements is apparently simple; they are easily isolated and manipulated *in vitro;* and since plasmids are thought to be dispensible, their manipulation does not appear to have adverse consequences to the hosts (del Solar et al., 1998). Plasmids have an essential region that contains the genes or loci involved in replication and its control and include: the origin(s) of replication which is characteristic of each replicon; a protein, Rep, involved in the initiation of replication; and plasmid-borne genes involved in the control of replication (del Solar et al., 1998). The majority of plasmids replicate via rolling circle or theta mechanisms and both of these mechanisms have been described in detail by del Solar et al. (1998) in a recent review and a summary from their review follows.

1.5.2 *Rolling circle replication.*

Briefly, replication by the rolling circle mechanism is unidirectional and is considered to be an asymmetric process because synthesis of the leading strand and synthesis of the lagging strand are uncoupled (Figure 1.4). Initiation of replication starts with the plasmid-encoded Rep protein, which introduces a site-specific nick on the plus strand, at a region called the double stranded origin (*dso*). The nick leaves a 3'-OH end that is used as a primer for leading strand synthesis and probably involves host replication proteins (Figure 1.4 A). Elongation from the 3'-OH end is accompanied by the displacement of the parental plus strand and continues until the replisome reaches the reconstituted *dso* (Figure 1.4 B). At this time a DNA strand transfer reaction takes place to terminate leading strand replication. The newly synthesized leading plus strand remains covalently bound to the same parental plus strand. Completion of leading strand replication results in the following end products: a double stranded (ds) DNA molecule consisting of the parental strand and the newly synthesized plus strand (Figure 1.4 C), and a single stranded (ss) DNA intermediate which corresponds to the parental plus strand (Figure 1.4 D). The parental plus strand is then converted into dsDNA form by host proteins such as RNA polymerase which initiates replication at the single stranded origin (*sso*), a site that is physically distinct from the *dso* (Figure 1.4 E). The last stage of rolling circle replication is the conversion of the ssDNA intermediate into a duplex plasmid molecule (Figure 1.4 F). Lagging strand synthesis initiates and terminates at the *sso* and this site is critical for plasmid stability. It has been shown that deletion of plasmid *sso* regions leads to a reduction in the plasmid copy number, accumulation of ssDNA molecules, and rapid loss of the plasmid in the absence of selective pressure (del Solar et al., 1998). Lagging strand replication initiates by RNA polymerase (RNAP) synthesis of a short RNA primer. The last step of rolling circle replication involves the supercoiling of the replication products by host DNA gyrases. Rolling circle replication is used by many small plasmids in gram positive bacteria.

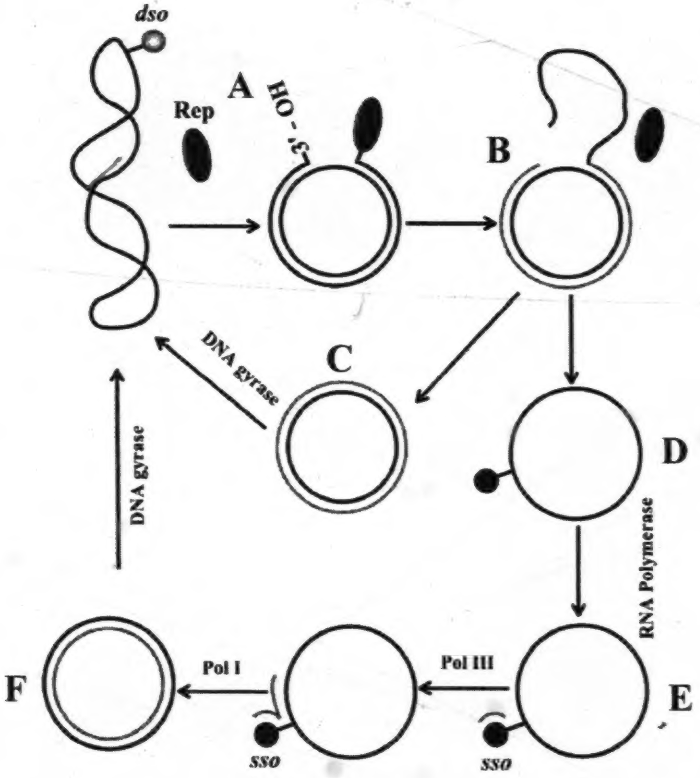

Figure 1.4. Schematic representation of rolling circle replication.
[*dso*, double stranded origin; *sso*, single stranded origin; Rep, replication protein; Pol I, DNA polymerase I; Pol III, DNA polymerase III. Leading strand shown in gray. Figure adapted from del Solar et al. (1998)].

1.5.3 *Theta-type replication.*

The second method of plasmid replication is of the theta type. Replication by the theta mechanism involves melting and opening of the parental strands at the origin of replication, *oriX,* and synthesis of an RNA primer, which begins replication by covalent extension of the primer RNA (Figure 1.5 A). DNA synthesis is continuous on one of the strands (leading strand) and discontinuous on the other (lagging strand), although synthesis of the two strands has been shown to be coupled (del Solar et al., 1998). Theta-type DNA synthesis can start from one or from several origins, and replication can be uni- or bi-directional. The replication intermediates are seen as typical θ ("theta") shaped molecules (Figure 1.5 B). If replication proceeds in one direction (uni-directional replication) then a single replication fork moves around the plasmid until it returns to the origin and then the two daughter plasmids separate. If replication proceeds in two directions (bi-directional replication) then two replication forks move out from the origin in opposite directions until they meet and replication is complete. The two daughter plasmids then separate (Figure 1.5 C).

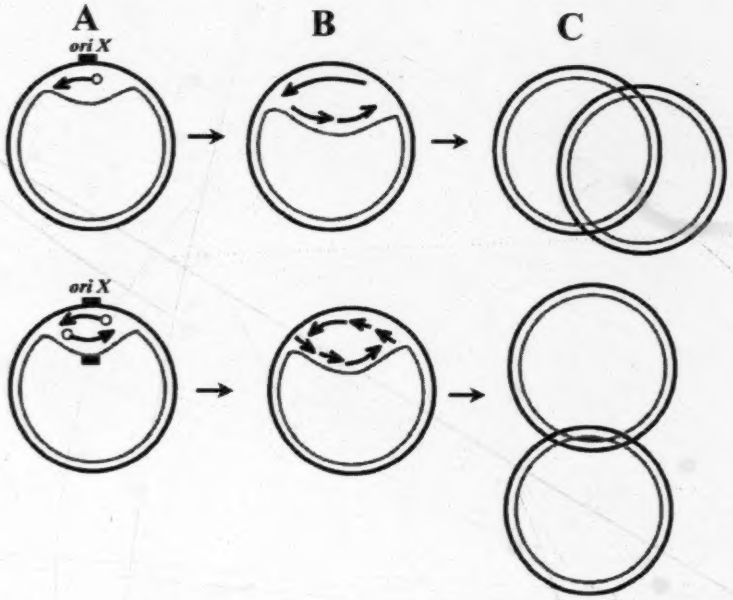

Figure 1.5. Theta-type plasmid replication showing unidirectional and bidirectional synthesis.

[The origin of replication is designated *ori*X. Figure adapted from Snyder and Champness (1997) and del Solar et al. (1998)].

Plasmids using the theta mechanism of replication require a plasmid-encoded Rep initiator protein. The origin of replication contains directly repeated sequences, termed iterons, which are the binding sites for the plasmid-encoded Rep proteins and these specific sequences have control properties in that they are not only essential for replication but also are key elements for the control of plasmid replication (del Solar et al., 1998). Many origins of theta-replicating plasmids include the following additional features: i) an adjacent AT-rich region containing sequence repeats, where opening of the strands and assembly of host initiation factors occur and ii) one or more sites (dnaA boxes) where the host DNA initiator protein binds (del Solar et al., 1998). The plasmid DNA sites are essential components of the origin of replication since they are required to organize a functional replisome. The theta mechanism is the most common form of DNA replication (Snyder and Champness, 1997).

1.5.4 *Factors affecting plasmid stability.*

The most important process for any bacterial plasmid is stable maintenance in the host cell (Wegrzyn and Wegrzyn, 2002) and plasmids must have mechanisms in place to ensure their presence in future generations of host cells. Plasmids must replicate often enough to produce copies in amounts that will allow for distribution to both daughter cells after division of the mother cell (Wegrzyn and Wegrzyn, 2002). To prevent energetic exhaustion of the host leading to cell death, the frequency of plasmid replication must not be too high. Plasmid molecules present in low copy numbers should be partitioned efficiently after each round of replication to ensure that they are present in each daughter cell (Wegrzyn and Wegrzyn, 2002). The emergence of plasmidless cells in a population of bacteria that originally carried plasmids is considered a serious disadvantage from the point of view of the plasmid, as cells devoid of plasmids usually grow faster than plasmid-bearing cells (Wegrzyn and Wegrzyn, 2002) and plasmid-bearing cells will eventually disappear from the population.

Plasmid replication plays an important role in stability and efficient control of plasmid replication is important since it sets the average copy number (Williams and Thomas, 1992). Although some plasmids are lost quite rapidly from bacterial cells when appropriate selective pressure is removed, some plasmids can be retained for many generations in the absence of any selective pressure and there are a number of mechanisms that contribute to the segregational stability of these plasmids. Some of the stability functions used by plasmids do not involve active partition mechanisms that actively distribute the plasmid molecules to the daughter cells. Instead, mechanisms that affect other aspects of plasmid life cycles such as the resolution of oligomers formed by recombination or the killing of plasmid-free cells by plasmid-encoded functions are used (Nordstrom and Austin, 1989).

High copy number plasmids are generally inherited efficiently on the basis of random segregation alone (Williams and Thomas, 1992). High copy number plasmids make use of a basic replicon and one or more helper elements that aid its random distribution. These helper elements include: i) site-specific recombination systems, ii) anticlumping systems, and iii) functions that improve copy number distribution (Nordstrom and Austin, 1989). In addition, plasmid multimer resolution functions maximize the number of independently segregating units at cell division (Williams and Thomas, 1992) thereby increasing the probability of segregation to daughter host cells.

Low copy number plasmids must have an active element to ensure plasmid stability. These active elements include: i) killer systems in which the plasmid codes for the production of a killer substance and an agent that blocks its activity and if the plasmid is lost the blocking agent decays but the killer substance persists and kills the host cell, and ii) partition systems which selectively move DNA molecules to ensure that each daughter cell receives at least one copy of the plasmid (Nordstrom and Austin, 1989). Often large plasmids have a low copy number to minimize the metabolic burden they place on the cell (Williams and Thomas, 1992) and these large plasmids must utilize active elements to ensure that they are maintained by the host cells after division. In addition, plasmid copy number control circuits often allow stimulation of plasmid replication when copy numbers fall thereby minimizing the number of cells which contain only one plasmid molecule prior to division (Williams and Thomas, 1992).

The mode of replication used by plasmids can also affect plasmid stability especially if foreign DNA is inserted through recombinant techniques. It has been demonstrated that recombinant rolling circle plasmids often have a high degree of instability due to the formation of high-molecular-weight (HMW) multimers whereas this does not occur with theta replicating plasmids (Kiewiet et al., 1993). HMW multimers have been implicated in both structural and segregational instability (Gruss and Ehrlich, 1988; Kiewiet et al., 1993). The nature of the inserted DNA can determine whether HMW multimers are generated but the site of insertion of the foreign DNA is not a determining factor in HMW multimer production (Gruss and Ehrlich, 1988). As little as 500 base pairs of DNA inserted into rolling circle plasmids can result in HMW multimer formation; however, as the size of the inserts increases, the proportion of HMW multimers also increases (Gruss and Ehrlich, 1988). It is generally believed that insert-induced HMW multimer formation results from nontermination of leading strand displacement during rolling circle replication (Kiewiet et al., 1993). Theta-type plasmids do not generate single stranded DNA replication intermediates and thus do not form HMW products and are often considered to be good candidates for the development of efficient and stable cloning vectors (Kiewiet et al., 1993).

1.5.5 *Use of plasmids as expression vectors.*

Plasmids are frequently used as vectors for the expression of heterologous gene products in LAB because of the ease of manipulation (Seegers, 2002). However, there are disadvantages associated with the use of plasmids as vectors: i) the copy number of plasmids may vary, ii) plasmids may be lost in the absence of selective pressure, usually in the form of antibiotic pressure, and iii) plasmids may be structurally unstable (Renault, 2002). Addressing these limitations is essential for the successful production of desired gene products. Some researchers consider integration into the chromosome to be a more reliable approach to stabilizing and maintaining the desired genes in the heterologous host (Henrich et al., 2002) because a chromosomally integrated gene is more stable in the absence of selective pressure than a plasmid-borne gene (Billman-Jacobe, 1996). However, even though integrated systems offer the benefit of greater genetic stability of the heterologous genes, the expression levels can be compromised (Seegers, 2002) due to a reduction in gene copy number (Billman-Jacobe, 1996). For example, there would be only one copy per host cell compared to multiple copies if high copy number plasmids are used. Researchers have shown that genes can be stably expressed in the absence of antibiotic pressure by heterologous LAB using plasmids as vectors. For example, the bacteriocin, mesentericin Y105, was heterologously produced by *Leuconostoc cremoris* at an amount similar to that of the wild-type producer strain using pTXL1, a small cryptic plasmid from *Lc. mesenteroides* subsp. *mesenteroides* Y110, that was developed into a food-grade vector (Biet et al., 2002). To conform to the food-grade criterion, plasmids must not contain genes that confer resistance to antibiotics and should consist of genetic elements derived from strains that have traditionally been used in food fermentations and are considered to be GRAS (Emond et al., 2001; Henrich et al., 2002). The replicon from another small, stable theta-replicating plasmid, pCD4, was used in a two-component food-grade cloning system for the stable transfer of a phage resistance mechanism into heterologous *Lactococcus lactis* strains (Emond et al., 2001). Researchers have also used the α-galactosidase gene from *L. raffinolactis* and the theta-replicon from a *L. lactis* plasmid to construct a novel food-grade cloning vector for introducing phage resistance into strains of *L. lactis* (Boucher et al., 2002). Thus, recombinant technology using plasmids as vectors is an attractive option because plasmids are easy to genetically manipulate, high copy number plasmids produce multiple copies of the bacteriocin genes, and there is an increasing amount of information on theta-type plasmids that are structurally and segregationally stable.

1.6 Promoters

1.6.1 *Promoters and transcription.*

In addition to using the appropriate expression vectors, the choice of promoters used in recombinant technology experiments could have a significant effect on the heterologous production of proteins and peptides by LAB. The search for efficient, versatile promoters is important to the development of expression systems utilizing bacteria (Billman-Jacobe, 1996) and many researchers are exploring the use of regulated and constitutive promoters to optimize gene expression in various strains. Constitutive promoters differ from regulated promoters in that they have in their sequences all the information necessary for specifying the transcriptional efficiency of their complexes with RNA polymerase, whereas regulated promoters require either positive or negative effectors in the form of inducers or repressors (Fournier et al., 1999).

Much of the basic information on promoters, RNA polymerases, and transcription that is available is based on *E. coli* as the model organism although in recent years there has been increasing amounts of information being gathered on promoters from gram positive organisms, such as *Bacillus subtilis* and *Lactococcus lactis*. Promoters are regions on the DNA strand that are recognized by RNA polymerases which then bind to these specific sites and begin the process of transcribing the DNA template into messenger RNA. The components of the promoter that are of primary importance for recognition and binding by the RNA polymerase are the –10 and –35 hexamers, the spacer region between these two hexamers, and a region between –40 and –60 that is known as the upstream (UP) region. The –10 and –35 regions consist of sequences that are conserved in *E. coli* as well as *Bacillus, Lactobacillus,* and *Streptococcus* spp. and are called consensus sequences. The consensus sequences are TTGACA (-35) and TATAAT (-10) and it is generally thought that the closer the –10 and –35 regions of a promoter match the consensus sequences, the better the promoter functions *in vitro* as well as *in vivo* (Ellinger et al., 1994; Harley and Reynolds, 1987; Hawley and McClure, 1983). A consensus length of 17 base pairs for the spacer region has also been established to result in promoters with more activity *in vitro* and *in vivo* than those promoters with either longer or shorter spacers (Harley and Reynolds, 1987; Hawley and McClure, 1983; Mitchell et al., 2003). The UP region at –40 to –60 base pairs upstream of the transcription start site is a very A + T-rich sequence that, in *E. coli,* is an additional determinant of promoter activity (Estrem *et al.*, 1998; Ross *et al.*, 1993; Ross *et al.*, 2001). In addition to these components other factors such as DNA conformation and electrostatic binding within the promoter can also affect promoter strength (Vanet et al., 1999).

1.6.2 *Promoter activity and strength.*

There are several components that can affect promoter activity making some promoters more active (stronger) than others. Several researchers have determined the effects of base substitutions in the –10 and –35 regions, on promoter strength. Kobayashi et al. (1990) constructed 18 variant *lac* UV5 promoters with single base substitutions in the –35 region and used a purified *E. coli* RNA polymerase to determine the effects of the substitutions on binding affinity to the RNA polymerase and the rate of open complex formation, two parameters that govern promoter strength. They found that: a) the consensus sequence, TTGACA, gave the highest values for both parameters, b) alteration in promoter strength is dependent on both the position and the base species of the substitution, c) base substitutions at the –34 position have a marked effect on both parameters, d) the cytosine at position –32 can not be replaced with other nucleotides without a significant reduction in promoter strength, and e) a base substitution at position –31 has only a small effect on the binding affinity of the RNA polymerase (Kobayashi et al., 1990). Fournier et al (1999) examined the effect of point mutations in the –10 and –35 regions using promoters for the β-lactamase gene from *Klebsiella oxytoca* which were cloned upstream of a *cat* (chloramphenicol acetyltransferase) gene and relative promoter strengths were determined in *E. coli* and *K. oxytoca.* They observed a 4- to 31-fold increase in chloramphenicol acetyltransferase activity for promoters with mutations compared to the wild-type promoter (Fournier et al., 1999). A transversion of G to T in the first base of the –10 sequence (GATAGT, wild-type) resulted in a 20-fold increase in promoter strength in *E. coli.* A transversion of G to A at position 5 of the –10 region or a transversion of T to A at position 4 of the –35 region (TTGTCA, wild-type) resulted in only a 4- to 9-fold increase. However, a promoter carrying both of these mutations resulted in a 15-fold increase in chloramphenicol acetyltransferase (CAT) expression in *E. coli* and a 61-fold increase in *K. oxytoca* compared expression with the wild-type promoter (Fournier et al., 1999). Fournier et al. (1999) also investigated the effect of the spacer region on promoter strength in the G to T mutant and found that a reduction from 17 base pairs to 16 base pairs resulted in a 9-fold decrease in activity. Recently, modifications were made to increase the rate of transcription of a *lacZ* gene in *Bacillus subtilis* by changing the –35 region (TACTAA) of the *aprE* promoter to make it identical to the consensus sequence while keeping the wild-type –10 region (TACAAT), which was almost identical to the consensus sequence, the same (Jan et al., 2001). Changing the four bases in the -35 region results in an increase in β-galactosidase activity of about 106-fold (Jan et al., 2001).

Point mutations in the essential TGTG motif of the spacer regions of *B. subtilis* α-amylase promoters were studied using *B. subtilis* and *E. coli* RNA polymerases (Voskuil and Chambliss, 1998). In a previous study the investigators found that a transversion of G to T at position –15 virtually eliminated α-amylase production in both *B. subtilis* and *E. coli* (Voskuil et al., 1995). A subsequent study showed that a transversion of G to C at position –15 reduced *in vitro* transcription by 95% and mutations at positions –18, -17, and –16 reduced activity to approximately 25% of the wild-type level whereas transversion of T to A at position –19 had essentially no effect (Voskuil and Chambliss, 1998). The importance of the TG motif in the spacer region was further studied by Burr et al. (2000) who conducted a systematic study of the effects of these base sequences immediately upstream of the –10 region using an *E. coli* promoter. They constructed derivatives with all possible combinations of bases at positions –15 and –14 and determined that promoter activity is greatest when T and G are at positions –15 and –14 and activity is further enhanced by a second T and G at positions –17 and –16 immediately upstream of the first TG motif (Burr et al., 2000). Promoter activity is critically dependent on the bases at positions –15 and –14 with substitution of the T at –15 having a lesser effect on activity than a substitution of the G at –14 (Burr et al., 2000). The investigators also determined that activity is optimal with a pyrimidine at position –17 and a purine at position –16 (Burr et al., 2000). A comparision of 554 *E. coli* promoter sequences showed that promoters containing the TG motif at positions -15/-14 had –10 and –35 hexamer sequences that were less well conserved than their non-TG counterparts (Mitchell et al., 2003). Comparison of TG with non-TG promoters showed that a higher proportion of TG promoters had a longer spacer region and that the TG motif is essential for the maximal activity of seven selected promoters (Mitchell et al., 2003). Promoters with poorer matches to the –10 and –35 consensus sequences were more dependent on the TG motif for optimal activity (Mitchell et al., 2003).

An increasing amount of work on promoters from LAB and the influence of promoter activity on gene expression in LAB has been conducted. Five native promoters from *L. lactis* subsp. *cremoris* Wg2 were isolated, characterized, and tested for activity in *B. subtilis, L. lactis* IL1403, and *E. coli* by van der Vossen et al. (1987). The promoters varied in their –10 and –35 sequences as well as their activity based on level of resistance to chloramphenicol and CAT activities. Higher chloramphenicol resistance and CAT activities were observed with those promoters that had either complete consensus in the –35 region or differed in just one nucleotide (van der Vossen et al., 1987). Four of the five promoters contained a TG sequence upstream of the –10 hexamer and P44, the promoter that was missing this TG sequence, showed lower activity (van der Vossen et al., 1987).

A library of synthetic promoters for *L. lactis* has been constructed in which an attempt was made to keep the consensus sequences constant while the sequences of the spacer regions were randomized (Jensen and Hammer, 1998). Thirty eight promoters were created with a wide range of activity that differed from 0.3 to more than 2,000 relative units using β-galactosidase genes as reporters (Jensen and Hammer, 1998). When these promoters were tested in *L. lactis,* the researchers determined that the weak promoters all had changes either in the consensus sequences or in the length of the spacer region and that clones with one base pair deletions or changes outside the –35 and –10 regions had activities that were within the range of the 'perfect' (-10 and –35 consensus sequences, 17 base pair spacer region) clones; therefore, consensus regions outside of the –35 and –10 sequences were determined to have little importance with respect to promoter strength in *L. lactis* (Jensen and Hammer, 1998). The investigators also found that some promoters that were strong in *L. lactis* were relatively weak when tested in *E. coli* and vice versa and that the pattern observed in *L. lactis* which was that the strong promoters were the 'perfect' ones did not hold true for *E. coli* (Jensen and Hammer, 1998). Solem and Jensen (Solem and Jensen, 2002) asserted that in *L. lactis* the actual sequence of bases in the spacer region is less important for the strength of a particular promoter than the resulting DNA structure and that by randomizing many base pairs simultaneously in the region between the –10 and –35 consensus sequences it is possible to change the DNA structure and the binding of transcription factors to the promoter sequences (Solem and Jensen, 2002). It is therefore important to consider that the strength of promoters is often context dependent (Solem and Jensen, 2002) and that the ranking of individual promoters according to strength may change when they are used in a different system or in a different organism as was demonstrated when the same promoters were used in *L. lactis* and *E. coli* resulting in different levels of gene expression (Jensen and Hammer, 1998).

1.6.3 *Promoters and gene expression.*

Attempts have been made by researchers to enhance heterologous gene expression using various promoters. Investigators have adopted two different approaches, the use of regulated promoters or the use of constitutive promoters, for the expression of heterologous genes. A variety of strong but well regulated promoters have been identified over the years for engineering expression systems in bacteria (Cases and de Lorenzo, 1998). Regulated systems have the advantage of controllable overproduction of proteins at desirable moments during cell growth and proteins that are deleterious to the cells can be made because only small amounts of these proteins will be produced during the uninduced state (Billman-Jacobe, 1996; Kuipers et al., 1997; Makrides, 1996). The nisin-controlled expression system developed for LAB has been used many times for the controlled overexpression of heterologous genes. For example, esterase activity was expressed by heterologous *L. lactis* when the *nisA* promoter upstream of the *estA* gene was induced with subinhibitory amounts of nisin A (Fernandez et al., 2000). The esterase activity in the strains containing the *estA* gene and *nisA* promoter was 28- to 170-fold higher after nisin induction compared to the uninduced controls, which had esterase activities comparable to the wild-type strain (Fernandez et al., 2000). Recent studies have shown that PepO activity was 10- to 25-fold higher in an induced *L. lactis* strain compared to the host strain when the *pepO* gene encoding an oligopeptidase from *Lactobacillus rhamnosus* was cloned downstream of the *nisA* promoter (Christensson et al., 2002). The *nisA* promoter has been shown to be effective for the controlled production of numerous other heterologous proteins such as *Staphylococcus hyicus* lipase, bovine rotavirus antigen, human papillomavirus antigen, and *Brucella abortis* antigen in *L. lactis* (Miyoshi et al., 2002) as well as being active in other gram positive bacteria (Eichenbaum et al., 1998). The use of regulated promoters and the development of expression systems such as the nisin-controlled system have shown much promise for the production of industrially important proteins.

In systems where regulation of gene expression is not necessary it may be advantageous to use constitutive promoters for heterologous protein production. The advantage of constitutive promoters is that they do not require inducing agents for activity. An expression system based on the *Lactobacillus brevis slpA* (S-layer protein gene) promoter region was constructed for use in various LAB with the expression of *gusA* (β-glucuronidase), *luc* (luciferase), and *pepN* (aminopeptidase) genes being analyzed (Kahala and Palva, 1999). The researchers determined that based on transcription levels, the efficiency of the *slpA* promoters was of the same order of magnitude in both *L. lactis* and *Lb. plantarum* whereas in *Lb. gasseri* the recognition of the promoters was weaker or the transcripts synthesized were more unstable (Kahala and Palva, 1999). The protein yields varied significantly with the different hosts and reporter genes suggesting that there was either instability of the reporter proteins or there were differences in translational efficiency (Kahala and Palva, 1999). The genes for various bacteriocins have been heterologously expressed by LAB using the constitutive *L. lactis* subsp. *cremoris* Wg2 promoter, P32 (van der Vossen et al., 1987). For example, enterocin A from *Enterococcus faecium* DPC1146 was produced by *L. lactis* IL1403 and *Ent. faecalis* OG1X (O'Keeffe et al., 1999), brochocin-C from *Brochothrix campestris* ATCC 43754 was produced by *Carnobacterium* spp. (McCormick et al., 1998), and colicin V from *E. coli* was produced by *Carnobacterium* spp. and *L. lactis* IL1403 (McCormick et al., 1999). In addition to native promoters, a library of synthetic constitutive promoters of varying strengths has been generated with the aim of achieving a constant level of gene expression throughout the growth of a culture (Jensen and Hammer, 1998). Solem and Jensen (2002) developed an approach for modulating gene expression using this library of synthetic promoters. They applied their approach to chromosomal genes of *L. lactis* using two strategies: 1) introduction of a new gene expressed from the promoter library, and 2) modulation of the expression of preexisting genes or operons (Solem and Jensen, 2002). Using strategy 1, the researchers modulated the phosphofructokinase (PFK) activity in *L. lactis* by fusing the entire *pfk* gene to a library of promoters which resulted in PFK activities of the clones of 1.4 to 11 times the wild-type activity (Solem and Jensen, 2002). With strategy 2, gene expression of the *las* operon containing the genes for lactate dehydrogenase (*ldh*), pyruvate kinase (*pk*), and phosphofructokinase (*pfk*) was modulated using the synthetic promoters and activities ranging from 0.5 to 3.5 times the wild-type levels were obtained with the modulation affecting all three enzymes to the same extent (Solem and Jensen, 2002). This approach will likely have application for other bacterial systems and gene products and has potential for industrial uses because inducing agents are unnecessary.

Selecting the appropriate expression vectors and promoters for use in a particular system is key to achieving optimal gene expression and heterologous protein production. Researchers now have numerous options: rolling circle or theta replicating plasmids, constitutive or regulated promoters, and native or synthetic promoters, to achieve desired results in various bacterial systems.

1.7 Research objectives

Bacteriocins have enormous potential to enhance the safety and extend the storage life of foods. Heterologous production of bacteriocins by desirable strains of LAB is an area of research that continues to grow. However, a number of problems such as low production levels, instability, inactivation, and the development of resistance have been encountered. The focus of this research has been to address the issues of plasmid stability and production levels.

Prior to the initiation of this study the heterologous production of brochocin-C had been achieved in *Carnobacterium* spp. However, preliminary work indicated that the expression vector used previously was not stable in the absence of selective pressure and that the heterologous production of brochocin-C was greatly reduced compared with the wild-type producer. In addition, the effect of refrigeration temperatures on expression vectors had not been investigated. These factors limit the practical use of these genetically enhanced organisms in food systems. To overcome these limitations, the objectives of this research were to:

1. obtain the stable production of brochocin-C in the absence of selective pressure
2. investigate the effect of temperature on the stability of theta-type plasmids
3. determine the influence of various LAB promoters on heterologous brochocin-C production
4. quantify brochocin-C gene expression under the control of various promoters in *C. maltaromaticum* LV17C

1.8 References

Abee, T., Krockel, L., and Hill, C. (1995) Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *Int J Food Microbiol* **28**: 169-185.

Allison, G.E., Worobo, R.W., Stiles, M.E., and Klaenhammer, T.R. (1995) Heterologous expression of the lactacin F peptides by *Carnobacterium piscicola* LV17. *Appl Environ Microbiol* **61**: 1371-1377.

Axelsson, L., and Holck, A. (1995) The genes involved in production of and immunity to sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. *J Bacteriol* **177**: 2125-2137.

Axelsson, L., Katla, T., Bjornslett, M., Eijsink, V.G., and Holck, A. (1998) A system for heterologous expression of bacteriocins in *Lactobacillus sake*. *FEMS Microbiol Lett* **168**: 137-143.

Biet, F., Berjeaud, J.M., Worobo, R.W., Cenatiempo, Y., and Fremaux, C. (1998) Heterologous expression of the bacteriocin mesentericin Y105 using the dedicated transport system and the general secretion pathway. *Microbiology* **144 (Pt 10)**: 2845-2854.

Biet, F., Cenatiempo, Y., and Fremaux, C. (2002) Identification of a replicon from pTXL1, a small cryptic plasmid from *Leuconostoc mesenteroides* subsp. *mesenteroides* Y110, and development of a food-grade vector. *Appl Environ Microbiol* **68**: 6451-6456.

Billman-Jacobe, H. (1996) Expression in bacteria other than *Escherichia coli*. *Curr Opin Biotechnol* **7**: 500-504.

Bolhuis, H., van Veen, H.W., Poolman, B., Driessen, A.J., and Konings, W.N. (1997) Mechanisms of multidrug transporters. *FEMS Microbiol Rev* **21**: 55-84.

Boucher, I., Parrot, M., Gaudreau, H., Champagne, C.P., Vadeboncoeur, C., and Moineau, S. (2002) Novel food-grade plasmid vector based on melibiose fermentation for the genetic engineering of *Lactococcus lactis*. *Appl Environ Microbiol* **68**: 6152-6161.

Brurberg, M.B., Nes, I.F., and Eijsink, V.G. (1997) Pheromone-induced production of antimicrobial peptides in *Lactobacillus*. *Mol Microbiol* **26**: 347-360.

Burr, T., Mitchell, J., Kolb, A., Minchin, S., and Busby, S. (2000) DNA sequence elements located immediately upstream of the -10 hexamer in *Escherichia coli* promoters: a systematic study. *Nucleic Acids Res* **28**: 1864-1870.

Cases, I., and de Lorenzo, V. (1998) Expression systems and physiological control of promoter activity in bacteria. *Curr Opin Microbiol* **1**: 303-310.

Christensson, C., Bratt, H., Collins, L.J., Coolbear, T., Holland, R., Lubbers, M.W., O'Toole, P.W., and Reid, J.R. (2002) Cloning and expression of an oligopeptidase, PepO, with novel specificity from *Lactobacillus rhamnosus* HN001 (DR20). *Appl Environ Microbiol* **68**: 254-262.

Cintas, L.M., Casaus, P., Havarstein, L.S., Hernandez, P.E., and Nes, I.F. (1997) Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. *Appl Environ Microbiol* **63**: 4321-4330.

Cleveland, J., Montville, T.J., Nes, I.F., and Chikindas, M.L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol* **71**: 1-20.

Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S., and Jones, D. (1987) Classification of *Lactobacillus divergens, Lactobacillus piscicola* and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus *Carnobacterium*. *Int J Syst Bacteriol* **37**: 310-316.

del Solar, G., Giraldo, R., Ruiz-Echevarria, M.J., Espinosa, M., and Diaz-Orejas, R. (1998) Replication and control of circular bacterial plasmids. *Microbiol Mol Biol Rev* **62**: 434-464.

Delves-Broughton, J., Blackburn, P., Evans, R.J., and Hugenholtz, J. (1996) Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek* **69**: 193-202.

Diep, D.B., Havarstein, L.S., and Nes, I.F. (1996) Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. *J Bacteriol* **178**: 4472-4483.

Duffes, F., Jenoe, P., and Boyaval, P. (2000) Use of two-dimensional electrophoresis to study differential protein expression in divercin V41-resistant and wild-type strains of *Listeria monocytogenes*. *Appl Environ Microbiol* **66**: 4318-4324.

Dykes, G.A., and Hastings, J.W. (1998) Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. *Lett Appl Microbiol* **26**: 5-8.

Economou, A. (1998) Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme. *Mol Microbiol* **27**: 511-518.

Economou, A. (1999) Following the leader: bacterial protein export through the Sec pathway. *Trends Microbiol* **7**: 315-320.

Eichenbaum, Z., Federle, M.J., Marra, D., de Vos, W.M., Kuipers, O.P., Kleerebezem, M., and Scott, J.R. (1998) Use of the lactococcal nisA promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. *Appl Environ Microbiol* **64**: 2763-2769.

Eijsink, V.G., Skeie, M., Middelhoven, P.H., Brurberg, M.B., and Nes, I.F. (1998) Comparative studies of class IIa bacteriocins of lactic acid bacteria. *Appl Environ Microbiol* **64**: 3275-3281.

Eijsink, V.G., Axelsson, L., Diep, D.B., Havarstein, L.S., Holo, H., and Nes, I.F. (2002) Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek* **81**: 639-654.

Ellinger, T., Behnke, D., Bujard, H., and Gralla, J.D. (1994) Stalling of *Escherichia coli* RNA polymerase in the +6 to +12 region *in vivo* is associated with tight binding to consensus promoter elements. *J Mol Biol* **239**: 455-465.

Emond, E., Lavallee, R., Drolet, G., Moineau, S., and LaPointe, G. (2001) Molecular characterization of a theta replication plasmid and its use for development of a two-component food-grade cloning system for *Lactococcus lactis*. *Appl Environ Microbiol* **67**: 1700-1709.

Ennahar, S., Sonomoto, K., and Ishizaki, A. (1999) Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. *J Bioscience Bioengineering* **87**: 705-716.

Ennahar, S., Deschamps, N., and Richard, J. (2000a) Natural variation in susceptibility of *Listeria* strains to class IIa bacteriocins. *Curr Microbiol* **41**: 1-4.

Ennahar, S., Sashihara, T., Sonomoto, K., and Ishizaki, A. (2000b) Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol Rev* **24**: 85-106.

Estrem, S.T., Gaal, T., Ross, W., and Gourse, R.L. (1998) Identification of an UP element consensus sequence for bacterial promoters. *Proc Natl Acad Sci U S A* **95**: 9761-9766.

Fernandez, L., Beerthuyzen, M.M., Brown, J., Siezen, R.J., Coolbear, T., Holland, R., and Kuipers, O.P. (2000) Cloning, characterization, controlled overexpression, and inactivation of the major tributyrin esterase gene of *Lactococcus lactis*. *Appl Environ Microbiol* **66**: 1360-1368.

Fournier, B., Gravel, A., Hooper, D.C., and Roy, P.H. (1999) Strength and regulation of the different promoters for chromosomal beta-lactamases of *Klebsiella oxytoca*. *Antimicrob Agents Chemother* **43**: 850-855.

Franke, C.M., Tiemersma, J., Venema, G., and Kok, J. (1999) Membrane topology of the lactococcal bacteriocin ATP-binding cassette transporter protein LcnC. Involvement of LcnC in lactococcin A maturation. *J Biol Chem* **274**: 8484-8490.

Franz, C.M., Worobo, R.W., Quadri, L.E., Schillinger, U., Holzapfel, W.H., Vederas, J.C., and Stiles, M.E. (1999) Atypical genetic locus associated with constitutive production of enterocin B by *Enterococcus faecium* BFE 900. *Appl Environ Microbiol* **65**: 2170-2178.

Franz, C.M., van Belkum, M.J., Worobo, R.W., Vederas, J.C., and Stiles, M.E. (2000) Characterization of the genetic locus responsible for production and immunity of carnobacteriocin A: the immunity gene confers cross-protection to enterocin B. *Microbiology* **146 (Pt 3)**: 621-631.

Fremaux, C., Hechard, Y., and Cenatiempo, Y. (1995) Mesentericin Y105 gene clusters in *Leuconostoc mesenteroides* Y105. *Microbiology* **141 (Pt 7)**: 1637-1645.

Gravesen, A., Jydegaard Axelsen, A.M., Mendes da Silva, J., Hansen, T.B., and Knochel, S. (2002) Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. *Appl Environ Microbiol* **68**: 756-764.

Gruss, A., and Ehrlich, S.D. (1988) Insertion of foreign DNA into plasmids from gram-positive bacteria induces formation of high-molecular-weight plasmid multimers. *J Bacteriol* **170**: 1183-1190.

Hammes, W.P., Weiss, N., and Holzapfel, W.H. (1992) The Genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes*. Balows, A. (ed). New York: Springer-Verlag, pp. 1535-1594.

Harley, C.B., and Reynolds, R.P. (1987) Analysis of *E. coli* promoter sequences. *Nucleic Acids Res* **15**: 2343-2361.

Hawley, D.K., and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res* **11**: 2237-2255.

Henrich, B., Klein, J.R., Weber, B., Delorme, C., Renault, P., and Wegmann, U. (2002) Food-grade delivery system for controlled gene expression in *Lactococcus lactis*. *Appl Environ Microbiol* **68**: 5429-5436.

Higgins, C.F. (1992) ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8**: 67-113.

Holo, H., Nilssen, O., and Nes, I.F. (1991) Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. *J Bacteriol* **173**: 3879-3887.

Horn, N., Martinez, M.I., Martinez, J.M., Hernandez, P.E., Gasson, M.J., Rodriguez, J.M., and Dodd, H.M. (1998) Production of pediocin PA-1 by *Lactococcus lactis* using the lactococcin A secretory apparatus. *Appl Environ Microbiol* **64**: 818-823.

Horn, N., Martinez, M.I., Martinez, J.M., Hernandez, P.E., Gasson, M.J., Rodriguez, J.M., and Dodd, H.M. (1999) Enhanced production of pediocin PA-1 and coproduction of nisin and pediocin PA-1 by *Lactococcus lactis*. *Appl Environ Microbiol* **65**: 4443-4450.

Huhne, K., Axelsson, L., Holck, A., and Krockel, L. (1996) Analysis of the sakacin P gene cluster from *Lactobacillus sake* Lb674 and its expression in sakacin-negative *Lb. sake* strains. *Microbiology* **142 (Pt 6)**: 1437-1448.

Jan, J., Valle, F., Bolivar, F., and Merino, E. (2001) Construction of protein overproducer strains in *Bacillus subtilis* by an integrative approach. *Appl Microbiol Biotechnol* **55**: 69-75.

Jensen, P.R., and Hammer, K. (1998) The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. *Appl Environ Microbiol* **64**: 82-87.

Kahala, M., and Palva, A. (1999) The expression signals of the *Lactobacillus brevis* slpA gene direct efficient heterologous protein production in lactic acid bacteria. *Appl Microbiol Biotechnol* **51**: 71-78.

Kiewiet, R., Kok, J., Seegers, J., Venema, G., and Bron, S. (1993) The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **59**: 358-364.

Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev* **12**: 39-85.

Kobayashi, M., Nagata, K., and Ishihama, A. (1990) Promoter selectivity of *Escherichia coli* RNA polymerase: effect of base substitutions in the promoter -35 region on promoter strength. *Nucleic Acids Res* **18**: 7367-7372.

Kuipers, O.P., de Ruyter, P.G., Kleerebezem, M., and de Vos, W.M. (1997) Controlled overproduction of proteins by lactic acid bacteria. *Trends Biotechnol* **15**: 135-140.

Leisner, J.J., Greer, G.G., and Stiles, M.E. (1996) Control of beef spoilage by a sulfide-producing *Lactobacillus sake* strain with bacteriocinogenic *Leuconostoc gelidum* UAL187 during anaerobic storage at 2 degrees C. *Appl Environ Microbiol* **62**: 2610-2614.

Luchansky, J.B. (1999) Overview on applications for bacteriocin-producing lactic acid bacteria. *Antonie Van Leeuwenhoek* **76**: 335.

Makrides, S.C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol Rev* **60**: 512-538.

McCormick, J.K., Worobo, R.W., and Stiles, M.E. (1996) Expression of the antimicrobial peptide carnobacteriocin B2 by a signal peptide-dependent general secretory pathway. *Appl Environ Microbiol* **62**: 4095-4099.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E., and Van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. *Appl Environ Microbiol* **64**: 4757-4766.

McCormick, J.K., Klaenhammer, T.R., and Stiles, M.E. (1999) Colicin V can be produced by lactic acid bacteria. *Lett Appl Microbiol* **29**: 37-41.

McMullen, L.M., and Stiles, M.E. (1996) Potential for use of bacteriocin-producing lactic acid bacteria in the preservation of meats. *J Food Prot* **Supplement**: 64-71.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. (1999) Food-related illness and death in the United States. *Emerg Infect Dis* **5**: 607-625.

Mitchell, J.E., Zheng, D., Busby, S.J., and Minchin, S.D. (2003) Identification and analysis of 'extended -10' promoters in *Escherichia coli*. *Nucleic Acids Res* **31**: 4689-4695.

Miyoshi, A., Poquet, I., Azevedo, V., Commissaire, J., Bermudez-Humaran, L., Domakova, E., Le Loir, Y., Oliveira, S.C., Gruss, A., and Langella, P. (2002) Controlled production of stable heterologous proteins in *Lactococcus lactis*. *Appl Environ Microbiol* **68**: 3141-3146.

Mora, D., Scarpellini, M., Franzetti, L., Colombo, S., and Galli, A. (2003) Reclassification of *Lactobacillus maltaromicus* (Miller et al. 1974) DSM 20342(T) and DSM 20344 and *Carnobacterium piscicola* (Collins et al. 1987) DSM 20730(T) and DSM 20722 as *Carnobacterium maltaromaticum* comb. nov. *Int J Syst Evol Microbiol* **53**: 675-678.

Mori, H., and Ito, K. (2001) The Sec protein-translocation pathway. *Trends Microbiol* **9**: 494-500.

Muriana, P.M. (1996) Bacteriocins for control of *Listeria* spp. in food. *J Food Prot* **Supplement**: 54-63.

Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink, V., and Holo, H. (1996) Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek* **70**: 113-128.

Nes, I.F., and Holo, H. (2000) Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers* **55**: 50-61.

Nilsen, T., Nes, I.F., and Holo, H. (1998) An exported inducer peptide regulates bacteriocin production in *Enterococcus faecium* CTC492. *J Bacteriol* **180**: 1848-1854.

Nordstrom, K., and Austin, S.J. (1989) Mechanisms that contribute to the stable segregation of plasmids. *Annu Rev Genet* **23**: 37-69.

Oliver, D.B. (1993) SecA protein: autoregulated ATPase catalysing preprotein insertion and translocation across the *Escherichia coli* inner membrane. *Mol Microbiol* **7**: 159-165.

Peck, M.W. (1997) *Clostridium botulinum* and the safety of refrigerated processed foods of extended durability. *Trends Food Sci Technol* **8**: 186-192.

Quadri, L.E., Yan, L.Z., Stiles, M.E., and Vederas, J.C. (1997) Effect of amino acid substitutions on the activity of carnobacteriocin B2. Overproduction of the antimicrobial peptide, its engineered variants, and its precursor in *Escherichia coli*. *J Biol Chem* **272**: 3384-3388.

Rasch, M., and Knochel, S. (1998) Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA-1 and bavaricin A. *Lett Appl Microbiol* **27**: 275-278.

Renault, P. (2002) Genetically modified lactic acid bacteria: applications to food or health and risk assessment. *Biochimie* **84**: 1073-1087.

Rhodehamel, E.J. (1992) FDA's concerns with sous vide processing. *Food Technol* **46**: 73-76.

Rodriguez, J.M., Martinez, M.I., Horn, N., and Dodd, H.M. (2003) Heterologous production of bacteriocins by lactic acid bacteria. *Int J Food Microbiol* **80**: 101-116.

Rose, N.L., Sporns, P., Stiles, M.E., and McMullen, L.M. (1999) Inactivation of nisin by glutathione in fresh meat. *J Food Sci* **64**: 759-762.

Rose, N.L., Palcic, M.M., Sporns, P., and McMullen, L.M. (2002) Nisin: A novel substrate for glutathione S-transferase isolated from fresh beef. *J Food Sci* **67**: 2288-2293.

Ross, R.P., Morgan, S., and Hill, C. (2002) Preservation and fermentation: past, present and future. *Int J Food Microbiol* **79**: 3-16.

Ross, W., Gosink, K.K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K., and Gourse, R.L. (1993) A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science* **262**: 1407-1413.

Ross, W., Ernst, A., and Gourse, R.L. (2001) Fine structure of *E. coli* RNA polymerase-promoter interactions: alpha subunit binding to the UP element minor groove. *Genes Dev* **15**: 491-506.

Schiebel, E., Driessen, A.J., Hartl, F.U., and Wickner, W. (1991) Delta mu H+ and ATP function at different steps of the catalytic cycle of preprotein translocase. *Cell* **64**: 927-939.

Seegers, J.F. (2002) Lactobacilli as live vaccine delivery vectors: progress and prospects. *Trends Biotechnol* **20**: 508-515.

Snyder, L., and Champness, W. (1997) Plasmids. In *Molecular Genetics of Bacteria*. Washington, D.C.: American Society for Microbiology, pp. 109.

Solem, C., and Jensen, P.R. (2002) Modulation of gene expression made easy. *Appl Environ Microbiol* **68**: 2397-2403.

Stiles, M.E. (1994) Potential for biological control of agents of foodborne disease. *Food Res Int* **27**: 245-250.

Stiles, M.E. (1996) Biopreservation by lactic acid bacteria. *Antonie Van Leeuwenhoek* **70**: 331-345.

Stiles, M.E., and Holzapfel, W.H. (1997) Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* **36**: 1-29.

Todd, E.C.D. (1989) Preliminary estimates of costs of foodborne disease in Canada and costs to reduce salmonellosis. *J Food Prot* **52**: 586-594.

Tomita, H., Fujimoto, S., Tanimoto, K., and Ike, Y. (1996) Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus* *faecalis* pheromone-responsive conjugative plasmid pYI17. *J Bacteriol* **178**: 3585-3593.

Vadyvaloo, V., Hastings, J.W., van der Merwe, M.J., and Rautenbach, M. (2002) Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. *Appl Environ Microbiol* **68**: 5223-5230.

van Belkum, M.J., and Stiles, M.E. (1995) Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. *Appl Environ Microbiol* **61**: 3573-3579.

van Belkum, M.J., Worobo, R.W., and Stiles, M.E. (1997) Double-glycine-type leader peptides direct secretion of bacteriocins by ABC transporters: colicin V secretion in *Lactococcus lactis*. *Mol Microbiol* **23**: 1293-1301.

van Belkum, M.J., and Stiles, M.E. (2000) Nonlantibiotic antibacterial peptides from lactic acid bacteria. *Nat Prod Rep* **17**: 323-335.

van der Vossen, J.M., van der Lelie, D., and Venema, G. (1987) Isolation and characterization *of Streptococcus cremoris* Wg2-specific promoters. *Appl Environ Microbiol* **53**: 2452-2457.

Vanet, A., Marsan, L., and Sagot, M.F. (1999) Promoter sequences and algorithmical methods for identifying them. *Res Microbiol* **150**: 779-799.

Voskuil, M.I., Voepel, K., and Chambliss, G.H. (1995) The -16 region, a vital sequence for the utilization of a promoter in *Bacillus subtilis* and *Escherichia coli*. *Mol Microbiol* **17**: 271-279.

Voskuil, M.I., and Chambliss, G.H. (1998) The -16 region of *Bacillus subtilis* and other gram-positive bacterial promoters. *Nucleic Acids Res* **26**: 3584-3590.

Wegrzyn, G., and Wegrzyn, A. (2002) Stress responses and replication of plasmids in bacterial cells. *Microb Cell Fact* **1**: 2.

Williams, D.R., and Thomas, C.M. (1992) Active partitioning of bacterial plasmids. *J Gen Microbiol* **138 (Pt 1)**: 1-16.

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C., and Stiles, M.E. (1995) A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. *J Bacteriol* **177**: 3143-3149.

Young, J., and Holland, I.B. (1999) ABC transporters: bacterial exporters-revisited five years on. *Biochim Biophys Acta* **1461**: 177-200.

2. Stable expression of brochocin-C by heterologous *Carnobacterium* spp.

2.1 Introduction

Biopreservation technology using lactic acid bacteria (LAB) and/or their bacteriocins can be used to introduce a hurdle to the growth of pathogens and spoilage bacteria in foods. Many researchers are focusing their efforts on achieving heterologous production of bacteriocins by selected strains of LAB. Recombinant DNA technology is being applied to enhance production of bacteriocins, to transfer bacteriocin genes to other species, and for mutation and selection of bacteriocin variants with increased and/or broader activity spectra (Abee et al., 1995). Work in our laboratory has focused on using plasmids to transfer bacteriocin gene products to heterologous LAB (Franz et al., 1999; McCormick et al., 1996; McCormick et al., 1998; McCormick et al., 1999; van Belkum et al., 1997).

Brochocin-C is a bacteriocin produced by *Brochothrix campestris* ATCC 43754, a non-pathogenic bacterium isolated from soil and grass (Siragusa and Cutter, 1993; Talon et al., 1988). This organism does not comprise the normal microflora of foods and would not be useful as a biopreservative in foods. Brochocin-C is active against a wide range of gram positive bacteria and inhibits the outgrowth of *Bacillus* and *Clostridium* spores, including spores of *C. botulinum* (McCormick et al., 1998). Brochocin-C is of great interest because its broad antibacterial spectrum appears to be equivalent to that of nisin (Stiles, 1996). Brochocin-C is a heat stable, two component bacteriocin and both peptides (BrcA and BrcB) are required for activity (McCormick et al., 1998). Previously, a brochocin-C construct was developed by fusing the nucleotides encoding the signal peptide of the bacteriocin, divergicin A, in front of the structural genes for BrcA and BrcB and ligating the immunity gene after the BrcB structural gene to allow export of the bacteriocin by the general protein secretion pathway (McCormick et al., 1998). This construct was ligated into the expression vector, pMG36e with transcription under control of the lactococcal P32 promoter to create pJKM67 (McCormick et al., 1998). This work allowed the expression of brochocin-C in heterologous hosts such as *Carnobacterium maltaromaticum* LV17C, *C. maltaromaticum* UAL26, and *C. divergens* NCIMB 702855 (McCormick et al., 1998). However, subsequent investigation revealed that the expression of brochocin-C was not stable unless the selective agent, erythromycin, was included in the growth medium. The necessity for the inclusion of an antibiotic negated the usefulness of these genetically enhanced bacteria in food applications.

The objective of this study was to develop an expression system that would permit the stable production of brochocin-C in the absence of antibiotic pressure. The approach was to use alternative expression vectors and study their suitability for the heterologous expression of brochocin-C. *Carnobacterium* spp. were chosen to serve as heterologous hosts because they comprise the natural microflora of food and are not typically associated with spoilage. In addition, some of the strains are wild-type producers of bacteriocins that could potentially act in a synergistic manner with brochocin-C.

2.2 Materials and methods

2.2.1 *Bacterial strains and culture conditions*

The bacterial strains and plasmids used in this study are listed in Table 2.1. *Carnobacterium* strains were grown in All Purpose Tween (APT) broth (Difco Laboratories, Becton Dickinson Microbiology Systems, Sparks, Maryland) at 25°C. *E. coli* was grown on a rotary shaker at 250 rpm at 37°C in Luria Bertani (LB) broth (Difco). APT agar plates were made by addition of 1.5% (wt vol^{-1}) agar (Difco) to broth media; soft media were prepared with 0.75% (wt vol^{-1}) agar. Erythromycin (Sigma-Aldrich Canada Ltd., Oakville, Ontario) at a concentration of 5 μg ml^{-1} for LAB and 200 μg ml^{-1} for *E. coli* was added as a selective agent when appropriate. Stock cultures of the bacterial strains were stored at -70°C in APT broth containing 20% (vol vol^{-1}) glycerol.

Table 2.1. Bacterial strains and plasmids used in this study.

Bacterial Strain or Plasmid	**Relevant Characteristics**[a]	**Reference or Source**
Strains		
Brochothrix campestris ATCC 43754	wild-type producer of brochocin-C	Siragusa and Cutter, 1993
E. coli XL1 Blue pTRKH2	contains pTRKH2	O'Sullivan and Klaenhammer, 1993
Carnobacterium maltaromaticum LV17C	cured dervivative of *C. maltaromaticum* LV17, bacteriocin-negative, plasmid free, brochocin-C sensitive	Ahn and Stiles, 1990b
C. maltaromaticum UAL26	produces piscicolin 126, brochocin-C sensitive	Our collection
C. divergens UAL278	bacteriocin-negative, brochocin-C sensitive	Our collection
Plasmids		
pMG36e	Em^R, 3.6kb	van de Guchte et al., 1989
pTRKH2	Em^R, 6.9kb	O'Sullivan and Klaenhammer, 1993
pCD11	derivative of pCD3.4, Em^R, 4.4 kb	Our collection
pJKM64	*Xba*I-*Kpn*I fragment from pJKM61 in pUC119, dvn::*brcA*, dvn::*brcB*, *brcI*, Ap^R, 3.9kb	McCormick et al., 1998
pVB1-4	*Xba*I-*Kpn*I fragment from pJKM64 in pMG36e, dvn::*brcA*, dvn::*brcB*, *brcI*, Em^R	This study
pVB2-4	*Sal*I-*Bgl*II fragment from pVB1-4 in pTRKH2, Em^R	This study
pCDB	*Eco*RV-*Fsp*I fragment from pVB2-4 in pCD11, Em^R	This study

[a] Ap^R, ampicillin resistant; Em^R, erythromycin resistant; dvn::*brcA*, brochocin-C peptide A gene fused to divergicin A signal peptide; dvn::*brcB*, brochocin-C peptide B gene fused to divergicin A signal peptide; *brcI*, brochocin-C immunity gene.

2.2.2 *DNA isolation and manipulation*

Large scale plasmid DNA preparations were done as described by Sambrook and Russell (2001) with the following modifications. Harvested and washed *E. coli* cells were resuspended in 8 ml of Solution I containing 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM glucose, and 4 mg ml^{-1} lysozyme (Sigma), incubated at 37°C for 15 min and subjected to alkali lysis. Harvested and washed carnobacteria cells were resuspended in 8 ml of Solution 1 containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM NaCl, 25% sucrose and 15 mg ml^{-1} lysozyme, incubated at 37°C for 30 min and subjected to alkali lysis with 3% SDS. Plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

Small scale plasmid isolations from carnobacteria were done as described by Sambrook and Russell (2001) with the following modifications. Harvested and washed cells were incubated at 37°C for 60 min in Solution I containing 15 mg ml^{-1} lysozyme. Lysis Solution II was added and followed by the addition of 150 μl of 3 M potassium acetate. DNA was precipitated overnight at -20°C in 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. Plasmid DNA was washed twice with 70% ethanol, dried under vacuum and resuspended in TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0)] with 0.1 mg ml^{-1} RNase. The solutions were incubated at 37°C for 30 min and plasmid DNA was stored at -20°C.

DNA fragments were recovered from agarose gels using QIAEX II (QIAGEN Inc., Mississauga, Ontario). Restriction endonucleases (Promega, Madison, Wisconsin) and T4 DNA ligase (New England Biolabs, Mississauga, Ontario) were used as recommended by the suppliers. Standard methods were used for restriction enzyme digestions, ligations and gel electrophoresis (Sambrook and Russell, 2001).

Competent *Carnobacterium* spp. were prepared according to the method described by van Belkum and Stiles (1995) with the following modifications. Cells were grown in APT broth supplemented with 4% (wt vol^{-1}) glycine, harvested and the cells were washed three times with ice-cold electroporation buffer (0.5 M sucrose, 2.5 mM $CaCl_2$). Prior to transformation, 40 μl of cell suspension was mixed with 1 μl of plasmid

DNA and held on ice for 15 min in a 0.2 cm electroporation cuvette. Transformation of *Carnobacterium* spp. by electroporation was performed using a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, California) set at 100Ω and 2.5kV. Immediately after electroporation, 1 ml of APT broth containing 0.5 M sucrose and 20 mM $MgCl_2$ was added to the cells that were then incubated at 25°C for 3 h. After incubation, the cells were plated on APT agar containing erythromycin. Plates were examined for transformants after incubation for 3 to 4 days at 25°C.

2.2.3 *Oligonucleotide synthesis and amplification reaction*

Two oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer (Applied Biosystems, Foster City, California) for use in amplification reactions. A 32-mer oligonucleotide (Cbn-C: 5'-TGAGAGATCTGAATTCGGTCCTCGGGATATGA-3') contained a *Bgl*II restriction site (underlined) and was based on the 5' end of the nucleotide sequence encoding an *Eco*RI restriction site and the P32 promoter in pVB1-4. A 33-mer oligonucleotide (Val-2: 5'-TATAGTCGACGGTACCTTTGTACTAGTTAGAGA-3') contained a *Sal*I restriction site (underlined) and was based on the 3' end of the nucleotide sequence encoding a *Kpn*I restriction site and the immunity gene for brochocin-C in pVB1-4. DNA was amplified in a 100 μl reaction mixture using a temperature cycler (OmniGene; InterSciences Inc., Markham, Ontario). PCR mixtures contained 1.0 μM of each primer, 200 μM deoxynucleotide triphosphates (Invitrogen Canada Inc., Burlington, Ontario), 2.5 mM $MgCl_2$, 2.5 U of *Taq*Plus Precision polymerase (Stratagene Cloning Systems, LaJolla, California) and 1X *Taq*Plus Precision buffer (Stratagene). The plasmid, pVB1-4, was used as template DNA for amplification. DNA was amplified with 30 cycles (denaturation, 94°C, 1 min; annealing, 60°C, 1 min; extension, 72°C, 1 min) followed by a final extension step at 72°C for 10 min.

2.2.4 *Construction of vectors for brochocin-C expression*

An expression vector, pVB1-4, was constructed to recreate pJKM67, a plasmid that was not available. This was done by excising the dvn:*brcA*, dvn:*brcB*, *brcI* construct from pJKM64 (McCormick et al., 1998) using the restriction endonucleases, *Kpn*I and *Xba*I and ligating the fragment into these same restriction sites in pMG36e, which contains the P32 promoter. The plasmid, pVB1-4, was created in much the same manner as pJKM67 (McCormick et al., 1998) except that the brochocic-C construct was excised from pJKM64 instead of pJKM61. The plasmid, pVB1-4, was transformed into competent cells of *C. maltaromaticum* LV17C and plated onto APT agar plates containing erythromycin. The production of brochocin-C by the clones was confirmed using the deferred inhibition assay (Ahn and Stiles, 1990a). The indicator strain used was *C. maltaromaticum* LV17C containing the plasmid, pMG36e, to ensure that the indicator strain was resistant to erythromycin. The plasmid, pVB1-4, was isolated using small scale plasmid extraction procedures. A theta-type plasmid, pTRKH2, was used for the expression of brochocin-C under the control of the P32 promoter by amplifying the brochocin-C construct from pVB1-4 with PCR using the primers, Cbn-C and Val-2. The PCR product was digested with the restriction endonucleases, *Bgl*II and *Sal*I, and the excised fragment was ligated into these sites in pTRKH2 to create pVB2-4. Competent cells of *C. maltaromaticum* LV17C were transformed with pVB2-4 and the clones were tested for resistance to erythromycin and for the production of brochocin-C. Large scale plasmid extraction of pVB2-4 was conducted and CsCl purified plasmid DNA was used to transform competent cells of *C. divergens* UAL278 and *C. maltaromaticum* UAL26. These clones were also tested for resistance to erythromycin and for the production of brochocin-C.

Another expression vector for brochocin-C was created using the theta-type plasmid, pCD11, by digesting the plasmid with *Eco*RV. The plasmid, pVB2-4, was digested with *Eco*RV and *Fsp*I, restriction sites upstream and downstream, respectively, of the brochocin-C genes and including the P32 promoter. The fragment containing the brochocin-C construct and P32 promoter was ligated into the *Eco*RV site of pCD11 and the resulting plasmid, pCDB, was used to transform *C. maltaromaticum* UAL26. Resistance to erythromycin and production of brochocin-C was determined. Large scale plasmid extraction of pCDB was conducted and CsCl purified DNA was used to transform competent cells of *C. maltaromaticum* LV17C and *C. divergens* UAL278. These organisms were also tested for resistance to erythromycin and production of brochocin-C.

2.2.5 *Segregational stability of pVB2-4 and production of brochocin-C*

The segregational stability of pVB2-4 and in *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26 or *C. divergens* UAL278 and the production of brochocin-C by these strains was determined during incubation at various temperatures. The percent segregational stability and the percentage of colonies producing brochocin-C reported was the average of three experiments. Frozen stock cultures of each of the three strains were grown overnight at 25°C in APT broth supplemented with erythromycin. The cultures were subcultured at least once more in antibiotic-containing broth prior to the initiation of the study. Fully grown cultures (ca. log 9 cfu ml^{-1}) were serially diluted using sterile 0.1% peptone water (Difco) and 100 µl of the 10^{-4} dilution was inoculated into 10 ml of fresh APT broth without erythromycin and incubated at 25°C until the cultures were fully grown (approximately 20 generations). These cultures were serially diluted in sterile 0.1% peptone water and 100 µl of a 10^{-4} dilution was inoculated into 10 ml of fresh APT broth and incubated at 25°C to obtain another 20 generations of growth. This procedure was repeated until approximately 100 generations was obtained. After every 20 generations, 100 µl of a 10^{-6} dilution from the fully grown culture was plated onto non-selective APT agar plates to obtain approximately 300 colonies per plate. To investigate the effect of temperature on plasmid stability, 1 ml of a 10^{-2} dilution from each of the three fully grown cultures was inoculated into 100 ml of fresh APT broth and incubated at 4, 6.5, and 11°C. At regular intervals 1 ml aliquots were removed, serially diluted in 0.1% peptone water, plated on APT agar plates, incubated at 25°C for 48 h and enumerated. Evaluating the number of erythromycin resistant colonies was carried out as described below.

The percentage of erythromycin resistant clones was determined by transferring 100 colonies (from APT agar plates containing 300 to 500 colonies) onto APT agar plates supplemented with erythromycin. The plates were incubated at 25°C for 48 h. The percent segregational stability was determined as the number of colonies out of 100 that were able to grow on the selective agar. The percentage of colonies producing brochocin-C was determined by transferring 50 colonies (from APT agar plates containing 300 to 500 colonies) onto APT agar plates. The plates were incubated at 25°C for 48 h. The agar plates were overlayed with soft APT agar containing a 1% inoculum of the indicator strains (*C. maltaromaticum* LV17C or *C. maltaromaticum* UAL26). The plates were incubated at 25°C for 24 h and examined for zones of inhibition.

The segregational stability of pTRKH2 in the *Carnobacterium* strains incubated at 4, 6.5, 11, and 25°C was also determined using the above protocol.

2.2.6 *Segregational stability of pCDB and production of brochocin-C*

The segregational stability of pCDB in *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26 or *C. divergens* UAL278 and the production of brochocin-C by these strains during incubation at 25 and 4°C were determined using the above protocol. The percent segregational stability and percentage of colonies producing brochocin-C reported for approximately 100 generations of growth was the average of two experiments.

The segregational stability of pCD11 in the *Carnbacterium* strains incubated at 25 and 4°C was also determined.

2.2.7 *Determination of brochocin-C activity*

The amount of heterologous brochocin-C activity produced by *Carnobacterium* spp. containing either pVB2-4 or pCDB was compared to production by the wild-type organism. The amount of detectable brochocin-C activity produced was determined using the spot-on-lawn method (Ahn and Stiles, 1990b). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution yielding a clear zone of inhibition on the indicator lawn and was multiplied by a factor of 100 to obtain the AU ml^{-1} of the original supernatant (Franz et al., 1996). Bacterial strains were grown in APT broth at 25°C. Erythromycin was included as a selective agent when appropriate. *C. maltaromaticum* LV17C was used as the indicator strain for determining brochocin-C activity by *Brochothrix campestris* ATCC 43754. *C. maltaromaticum* LV17C containing either pTRKH2 or pCD11 was used as the indicator strain for determining brochocin-C activity produced by *Carnobacterium* spp. containing pVB2-4 or pCDB, respectively.

2.3 Results and discussion

2.3.1 *Construction of expression vectors for brochocin-C*

The plasmid, pVB1-4, is a derivative of the lactococcal expression vector, pMG36e (van de Guchte et al., 1989) and transcription of the bacteriocin genes was under the control of the P32 promoter. PCR amplification of pVB1-4 resulted in a PCR product containing the nucleotide sequence encoding the P32 promoter, the two bacteriocin structural genes each of which was fused behind the divergicin A signal peptide and the brochocin-C immunity gene as well as two new restriction endonuclease sites, *Bgl*II and *Sal*I. Insertion of the brochocin-C construct, consisting of the P32 promoter and the genes for production, immunity and export, into pTRKH2 resulted in pVB2-4. The expression vector, pTRKH2 was chosen because of its reported segregational stability in LAB (O'Sullivan and Klaenhammer, 1993). The plasmid, pCDB, a derivative of pCD11 (van Belkum and Stiles, 1999), contained the genetic determinants for the P32 promoter, brochocin-C production and immunity, and divergicin A production and immunity.

2.3.2 *Stability of pVB2-4 and pCDB and production of brochocin-C in* Carnobacterium *spp.*

The stability of pVB2-4 and pTRKH2 in *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26, and *C. divergens* UAL278 grown in APT broth incubated at 25°C was determined. The segregational stability of pVB2-4 in *C. maltaromaticum* LV17C (Figure 2.1A) and *C. maltaromaticum* UAL26 (Figure 2.1B) at 25°C was 100% over 100 generations. In contrast, *C. maltaromaticum* LV17C (Figure 2.1A) and *C. maltaromaticum* UAL26 (Figure 2.1.B) containing the vector, pTRKH2, showed a steady decrease in plasmid stability with approximately 20% of the colonies able to grow on erythromycin-containing agar after 100 generations of growth. The segregational stability of pVB2-4 in *C. divergens* UAL278 (Figure 2.1.C) decreased rapidly after 40 generations with only 3% of the colonies having the ability to grow in erythromycin-containing agar after 100 generations of growth. In *C. divergens* UAL278 (Figure 2.1.C), the segregational stability of pTRKH2 was greater than that of pVB2-4 with approximately 55% of the colonies maintaining the ability to grow on selective agar. The percentage of colonies showing brochocin-C production corresponded with plasmid stability in all three strains containing pVB2-4. For *C. maltaromaticum* LV17C and UAL26 containing pVB2-4, 100% of the colonies tested produced zones of inhibition against the indicator strains for over 100 generations of growth (data not shown). For *C. divergens* UAL278 containing pVB2-4, the loss of brochocin-C production corresponded with the loss of plasmid stability with approximately 3% of the colonies tested producing zones of inhibition against the indicator strains after 100 generations of growth (data not shown). The insertion of the genetic determinants for brochocin-C appeared to have an advantageous effect on plasmid stability in *C. maltaromaticum* LV17C and UAL26 grown in APT broth at 25°C. It is possible that the production of brochocin-C by strains containing pVB2-4 provides enough selective pressure to ensure that the strains maintain the plasmid. Plasmid-free cells would not have the genes for immunity and would therefore be susceptible to inhibition by brochocin-C. However, this phenomenon was not observed for *C. divergens* UAL278. The presence of the brochocin-C construct

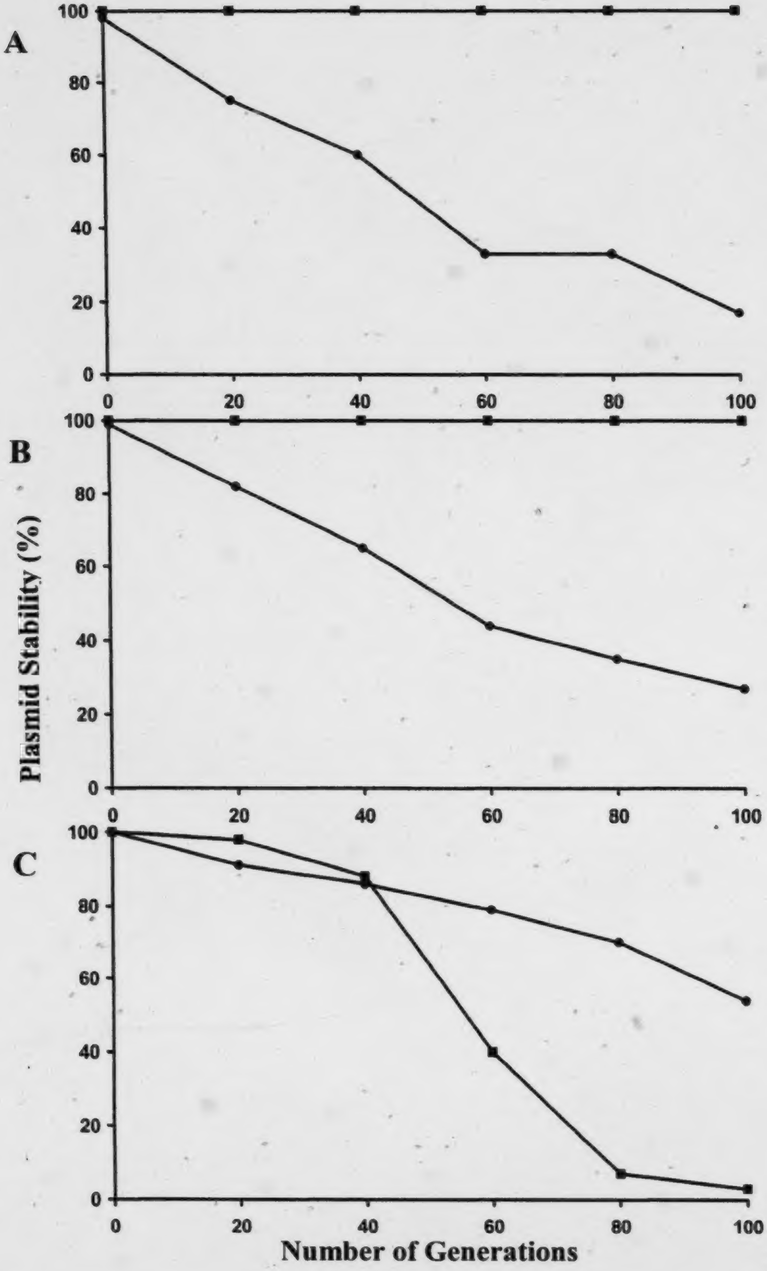

Figure 2.1. Segregational stability of pVB2-4 (■) and pTRKH2 (●) in *C. maltaromaticum* LV17C (**A**); *C. maltaromaticum* UAL26 (**B**); and *C. divergens* UAL278 (**C**) grown in APT broth incubated at 25°C. Percent plasmid stability is the number of erythromycin resistant colonies out of 100.

resulted in faster plasmid loss for pVB2-4 compared with pTRKH2. The insertion of additional genetic material in the form of the brochocin-C construct may place additional metabolic burden on *C. divergens* UAL278 resulting in faster plasmid loss. *C. divergens* UAL 278 may also be less sensitive to small amounts of brochocin-C than *C. maltaromaticum* spp. and therefore production of brochocin-C would not result in plasmid maintenance. It is also possible that plasmid stability is strain dependent with pVB2-4 being more stable in *C. maltaromaticum* spp. than in *C. divergens* UAL278. C. *maltaromaticum* LV17C and *C. maltaromaticum* UAL26 containing pVB2-4 could potentially be used as biopreservatives in food products stored at ambient temperatures. Reduced plasmid stability and resulting loss of brochocin-C production by *C. divergens* UAL278 limits the use of this organism in foods with extended storage potential.

The growth of the three strains and corresponding plasmid stability of pVB2-4 during incubation at 4, 6.5 and 11°C was investigated to determine the effect of low temperatures on stability and production of brochocin-C. The plasmid, pVB2-4, was more stable at 11°C than at 4 and 6.5°C in *C. maltaromaticum* LV17C (Figure 2.2.A) and *C. maltaromaticum* UAL26 (Figure 2.2.B). Plasmid stability dramatically decreased in both strains after 3 days of incubation at 6.5°C with only about 3% segregational stability remaining on day 5, and after 5 days of incubation at 4°C with approximately 10% of the colonies retaining the ability to grow in erythromycin-containing agar by day 7. The growth of the strains appeared to correspond with the loss of plasmid stability. At 4°C, plasmid stability dramatically decreased after 5 days incubation and a corresponding increase in bacterial numbers was observed at this time. Similar results were observed for the cultures incubated at 6.5°C with decreased plasmid stability and increased numbers of bacteria after 3 days of incubation. The presence of pTRKH2 containing the brochocin-C construct may place a stress on these strains during incubation at low temperatures thereby influencing growth rate. As plasmid stability decreased and this additional genetic material was lost, the strains appeared to increase their growth rates. Incubation at 11°C did result in a gradual decrease in plasmid stability over the duration of the incubation period. Approximately 65 and 85% of *C. maltaromaticum* LV17C and

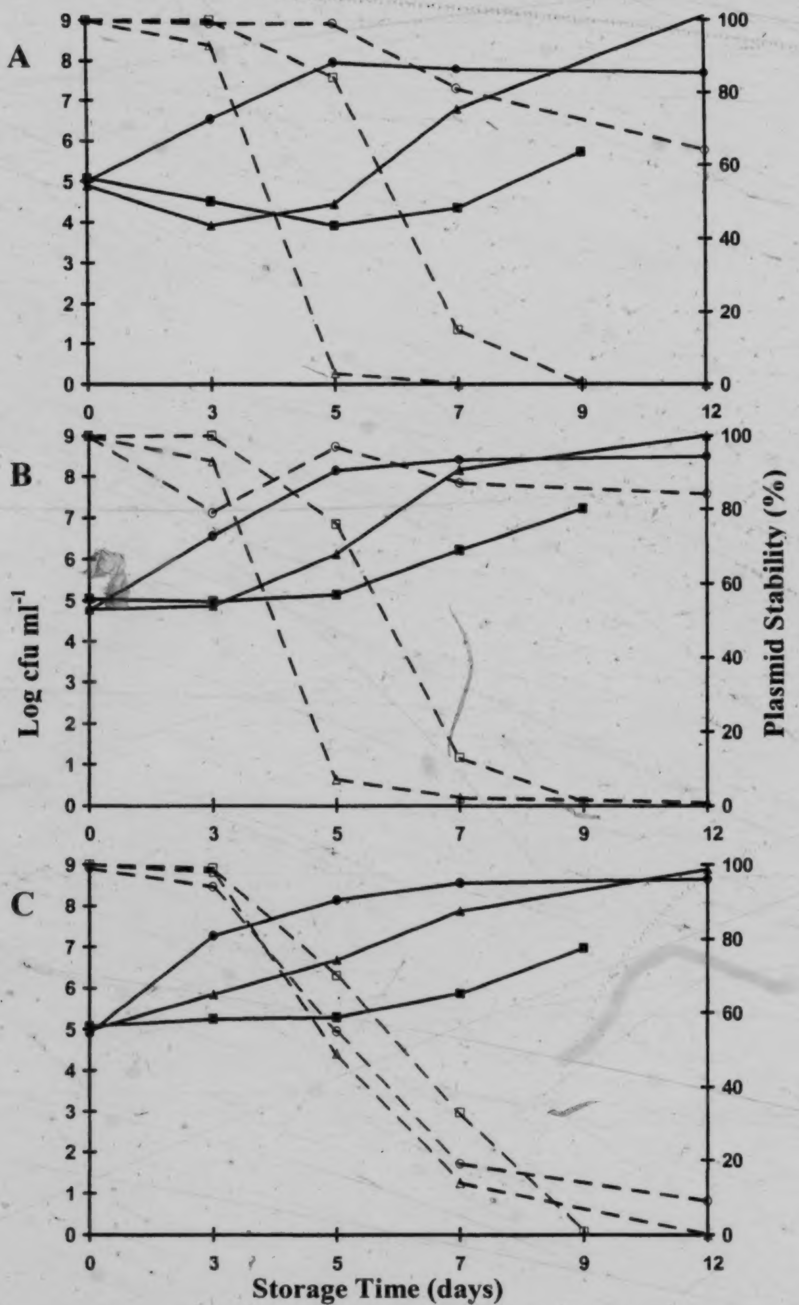

Figure 2.2. Growth (closed symbols) of *C. maltaromaticum* LV17C (**A**); *C. maltaromaticum* UAL26 (**B**); and *C. divergens* UAL278 (**C**) in APT broth and segregational stability (open symbols) of pVB2-4 during incubation at 4°C (■), 6.5°C (▲), and 11°C (●).

C. maltaromaticum UAL26, respectively, retained pVB2-4 after 12 days of incubation. Incubation at 11°C did not inhibit the growth of the strains containing pVB2-4. Temperature did not appear to have as dramatic an effect on *C. divergens* UAL278 containing pVB2-4 (Figure 2.2.C) with plasmid stability beginning to decrease after 3 days of incubation. Between 50 and 55% of *C. divergens* UAL278 retained pVB2-4 after 5 days of incubation at 6.5 and 11°C, respectively. At 4 °C, plasmid stability was approximately 70% after 5 days of incubation. Substantial loss of pVB2-4 was not observed until after 7 days of incubation. Increases in bacterial numbers were observed after 3 days incubation at 6.5 and 11°C and after 5 days incubation at 4°C. The plasmid, pVB2-4, was much less stable in *C. divergens* UAL278 than in *C. maltaromaticum* LV17 and UAL26 with only 10% of the colonies retaining the plasmid after 12 days incubation at 11°C compared to 65 and 85% stability in *C. maltaromaticum* LV17C and UAL26, respectively.

In all cases, incubation of the *Carnobacterium* strains at refrigeration temperatures dramatically decreased the stability of pVB2-4 with a corresponding loss of bacteriocin production. *C. maltaromaticum* LV17C and UAL26 containing pVB2-4 may have potential in the practical application of biopreservatives to enhance the safety and reduce the spoilage of foods stored at higher temperatures but due to the unstable nature of the vector their usefulness in minimally processed refrigerated foods with extended durability is unlikely.

The influence of temperature on the percent segregational stability and growth of *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26, and *C. divergens* UAL278 containing pTRKH2 was also tested to determine if insertion of the brochocin-C construct was responsible for plasmid instability or if pTRKH2, itself, was unstable when strains were incubated at low temperatures. The loss of plasmid stability for *C. maltaromaticum* LV17C and UAL26 containing pTRKH2 was faster compared to the same strains containing pVB2-4 at all three incubation temperatures. A dramatic decrease in plasmid stability was observed for *C. maltaromaticum* LV17C (Figure 2.3.A) incubated for 3 days at 4 and 6.5°C with approximately 10% of the colonies maintaining

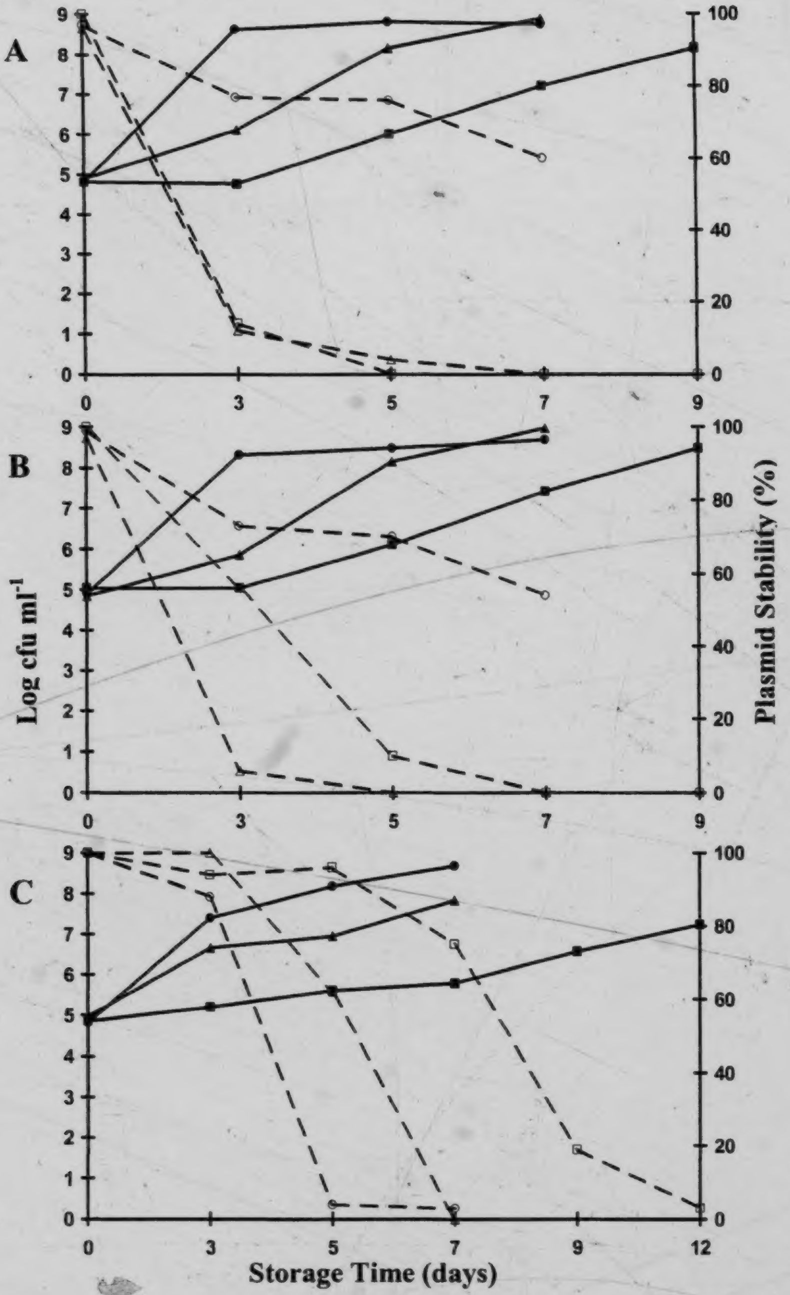

Figure 2.3. Growth (closed symbols) of *C. maltaromaticum* LV17C (**A**); *C. maltaromaticum* UAL26 (**B**); and *C. divergens* UAL278 (**C**) in APT broth and segregational stability (open symbols) of pTRKH2 during incubation at 4°C (■), 6.5°C (▲), and 11°C (●)

the ability to grow on erythromycin-containing agar. The percent stability was also lower for pTRKH2 during incubation at 11°C with approximately 60% stability at 7 days of incubation (Figure 2.3.A) compared to 80% stability for pVB2-4 at the same incubation time (Figure 2.2.A). Plasmid loss in *C. maltaromaticum* UAL26 was also faster with pTRKH2 compared to pVB2-4 at all three incubation temperatures with only 6% plasmid stability after 3 days incubation at 6.5°C, 10% stability after 5 days incubation at 4°C, and 50% stability after 7 days incubtion at 11°C (Figure 2.3.B). These results reflected the phenomonen observed at 25°C where the presence of the brochocin-C construct prolongs plasmid stability in *C. maltaromaticum* LV17C and UAL26. At 4°C, growth of the strains was observed once plasmid loss was detected. Incubation temperatures of 6.5 and 11°C did not appear to inhibit growth of *C. maltaromaticum* LV17C and UAL26 containing pTRKH2. In contrast to the results observed for *C. maltaromaticum* LV17C and UAL26, plasmid stability for pTRKH2 was higher in *C. divergens* UAL278 incubated at 4°C as compared to stability during incubation at 6.5 and 11°C (Figure 2.3.C). Loss of plasmid stability was also detected sooner with pTRKH2 compared to pVB2-4 in *C. divergens* UAL278 grown at 6.5 and 11°C. Less than 3% of the colonies were observed to maintain the ability to grow on selective agar by 7 days of incubation for *C. divergens* UAL278 containing pTRKH2 (Figure 2.3.C) compared to approximately 15% of the colonies showing growth on erythromycin-containing agar for *C. divergens* UAL278 containing pVB2-4 (Figure 2.2.C). The presence of the brochocin-C construct appeared to delay plasmid loss in *C. divergens* UAL 278 only during incubation at 6.5 and 11°C. Loss of plasmid stability during incubation at 4°C occurred sooner for pVB2-4 in *C. divergens* UAL278 compared to pTRKH2 in *C. divergens* UAL278.

Due to the failure of pVB2-4 to be maintained in *Carnobacterium* spp. during incubation at refrigeration temperatures, the stability of a new expression vector, pCD11, was investigated. Plasmid, pCD11, containing an erythromycin resistance marker gene, is a derivative of pCD3.4, a plasmid isolated from *C. divergens* NCIMB 702855 (Worobo et al., 1995). The plasmid has been fully characterized and contains several unique cloning sites (van Belkum and Stiles, 1999). The brochocin-C construct including the P32 promoter was inserted into the *Eco*RV site in pCD11 to create pCDB. Unlike pVB2-4 and pTRKH2 in *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26, and *C. divergens* UAL278 when incubated at refrigeration temperatures, pCDB had excellent segregational stability in all three strains during incubation at 4°C. There was no loss of plasmid stability or brochocin-C production with 100% of the colonies retaining the ability to grow on erythomycin-containing agar and 100% of the colonies produced zones of inhibition against the indicator strains for over 100 generations of growth (data not shown). The three strains containing pCDB also produced brochocin-C and no loss of segregational stability was observed during incubation at 25°C (data not shown). The segregational stability of pCD11 was also determined in *C. maltaromaticum* LV17C, *C. maltaromaticum* UAL26, and *C. divergens* UAL278 during incubation in APT broth at 4 and 25°C to determine whether the plasmid itself was stable in *Carnobacterium* spp. incubated at these temperatures or whether the presence of the brochocin-C construct enhanced stability as was observed with pVB2-4. The plasmid, pCD11, had excellent stability at both incubation temperatures with 100% of the colonies maintaining the ability to grow on selective agar over 100 generations of growth (data not shown). The development of pCDB allowed the stable expression of brochocin-C in *Carnobacterium* spp. during extended incubation at 4 and 25°C. These genetically enhanced LAB hold promise for use in food products stored at refrigeration temperatures.

2.3.3 *Brochocin-C activity*

Brochocin-C activity by heterologous *Carnobacterium* spp. was compared to activity produced by *B. campestris* ATCC 43754 using the spot-on-lawn assay. Brochocin-C activity was 8-fold less from *C. maltaromaticum* LV17C and *C. divergens* UAL278 containing pVB2-4 or pCDB compared to activity by the wild-type producer (Table 2.2). Activity from *C. maltaromaticum* UAL26 containing pVB2-4 or pCDB was 4-fold less than brochocin-C activity produced by *B. campestris* ATCC 43754 (Table 2.2). Production of brochocin-C by heterologous *Carnobacterium* spp. via the Sec

Table 2.2. Production of brochocin-C by *Brochothrix campestris* ATCC 43754 and heterologous *Carnobacterium* spp.

Bacterial strain	**Brochocin-C activity (AU ml^{-1})[a]**
B. campestris ATCC 43745	3200
C. maltaromaticum LV17C + pVB2-4	400
C. maltaromaticum UAL26 + pVB2-4	800
C. divergens UAL278 + pVB2-4	400
C. maltaromaticum LV17C + pCDB	400
C. maltaromaticum UAL26 + pCDB	800
C. divergens UAL278 + pCDB	400

[a] AU, arbitrary units

pathway results in a substantial reduction in detectable bacteriocin activity. This reduction in activity could potentially reduce the effectiveness of brochocin-C as a biopreservative in foods.

Brochocin-C has potential for use in biopreservation technology to enhance the safety and reduce the spoilage of foods. However, brochocin-C is not easily purified in large amounts (McCormick et al., 1998) and is naturally produced by *B. campestris*, an organism not associated with food. Therefore, it is desirable to obtain expression of brochocin-C in a suitable LAB. This was accomplished through previous work performed in our laboratory (McCormick et al., 1998). Subsequent studies revealed that the stable expression of brochocin-C could not occur in the absence of antibiotics in the growth medium. This necessitated investigating expression vectors to stably express brochocin-C in LAB hosts. *Carnobacterium* spp. were chosen as suitable hosts because they are less likely than some other LAB to cause spoilage of inoculated meat products and they frequently comprise the natural microflora of packaged, refrigerated meats. *C. maltaromaticum* LV17C and *C. maltaromaticum* UAL26 grow at refrigeration temperatures and *C. maltaromaticum* UAL26 produces a bacteriocin, piscicolin 126, with a broad antibacterial spectrum (Stiles and Hastings, 1991) which potentially could have a synergistic effect with brochocin-C. *C. divergens* UAL278 is also able to grow at refrigeration temperatures but has unusually high heat resistance in meat in that it is able to withstand heat treatment at 70°C for 2 minutes (McCormick et al., unpublished data). *C. divergens* UAL278 that has been genetically enhanced to produce a broad spectrum bacteriocin, such as brochocin-C, could be especially useful in the biopreservation of heat treated food products. Two theta-type plasmids, pTRKH2 and pCD11, were investigated as vectors for the heterologous expression of brochocin-C by *Carnobacterium* spp. Plasmid, pTRKH2, containing the brochocin-C construct was very stable in the absence of erythromycin in *C. maltaromaticum* LV17C and UAL26 incubated at 25°C but stability was dramatically decreased at refrigeration temperatures. Plasmid, pCD11, containing the brochocin-C construct was also very stable in the absence of antibiotic pressure as well as being stable at 25 and 4°C.

The objective of this study has been met by achieving the stable, heterologous production of brochocin-C in the absence of antibiotic pressure during incubation at ambient and refrigeration temperatures. However, as a result of the findings of this study other areas of research need to be investigated. Future studies should be conducted to assess the effect of low temperature on other theta-type plasmids that have potential for use as expression vectors. In addition, a potential problem associated with the production of brochocin-C by heterologous *Carnobacterium* spp. is reduced detectable activity compared with production by the wild-type organism. Possible reasons for decreased production include export of brochocin-C by the general secretory pathway rather than by a dedicated transport mechanism or reduced transcription of the brochocin-C genes using the lactococcal P32 promoter. Additional studies to investigate strategies to enhance heterologous production of brochocin-C by *Carnobacterium* spp. should be performed.

2.4 References

Abee, T., Krockel, L., and Hill, C. (1995) Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *Int J Food Microbiol* **28**: 169-185.

Ahn, C., and Stiles, M.E. (1990a) Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. *J Appl Bacteriol* **69**: 302-310.

Ahn, C., and Stiles, M.E. (1990b) Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. *Appl Environ Microbiol* **56**: 2503-2510.

Franz, C.M., Worobo, R.W., Quadri, L.E., Schillinger, U., Holzapfel, W.H., Vederas, J.C., and Stiles, M.E. (1999) Atypical genetic locus associated with constitutive production of enterocin B by *Enterococcus faecium* BFE 900. *Appl Environ Microbiol* **65**: 2170-2178.

McCormick, J.K., Worobo, R.W., and Stiles, M.E. (1996) Expression of the antimicrobial peptide carnobacteriocin B2 by a signal peptide-dependent general secretory pathway. *Appl Environ Microbiol* **62**: 4095-4099.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E., and Van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. *Appl Environ Microbiol* **64**: 4757-4766.

McCormick, J.K., Klaenhammer, T.R., and Stiles, M.E. (1999) Colicin V can be produced by lactic acid bacteria. *Lett Appl Microbiol* **29**: 37-41.

O'Sullivan, D.J., and Klaenhammer, T.R. (1993) High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene* **137**: 227-231.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Siragusa, G.R., and Cutter, C.N. (1993) Brochocin-C, a new bacteriocin produced by *Brochothrix campestris*. *Appl Environ Microbiol* **59**: 2326-2328.

Stiles, M.E., and Hastings, J.W. (1991) Bacteriocin production by lactic acid bacteria: potential for use in meat preservation. *Trends Food Sci Technol* **2**: 247-251.

Stiles, M.E. (1996) Biopreservation by lactic acid bacteria. *Antonie Van Leeuwenhoek* **70**: 331-345.

Talon, R., Grimont, P.A.D., Grimont, F., Gasser, F., and Boeufgras, J.M. (1988) *Brochothrix campestris* sp. nov. *Int J Syst Bacteriol* **38**: 99-102.

van Belkum, M.J., and Stiles, M.E. (1995) Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. *Appl Environ Microbiol* **61**: 3573-3579.

van Belkum, M.J., Worobo, R.W., and Stiles, M.E. (1997) Double-glycine-type leader peptides direct secretion of bacteriocins by ABC transporters: colicin V secretion in *Lactococcus lactis*. *Mol Microbiol* **23**: 1293-1301.

van de Guchte, M., van der Vossen, J.M., Kok, J., and Venema, G. (1989) Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. *Appl Environ Microbiol* **55**: 224-228.

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C., and Stiles, M.E. (1995) A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. *J Bacteriol* **177**: 3143-3149.

3. Effect of low temperature on stability of theta-type plasmids in *Carnobacterium maltaromaticum*

3.1 Introduction

Lactic acid bacteria (LAB) have a long history of use by man for the fermentation and preservation of foods. In recent years LAB have also been used as live vaccine carriers (Langella and Le Loir, 1999; Ribeiro et al., 2002; Seegers, 2002) and to heterologously produce proteins such as bovine β-lactoglobulin (Bernasconi et al., 2002) and bovine plasmin (Arnau et al., 1997). The heterologous production of bacteriocins such as brochocin-C (McCormick et al., 1998) and pediocin PA-1 (Horn et al., 1998) by LAB have enormous potential application as food biopreservatives. Plasmids have been used as vectors for the genes encoding these useful peptides and proteins. However, it has been recognized that there are drawbacks associated with the use of recombinant plasmids such as structural instability and variable copy numbers which can affect the maintenance and expression of cloned genes (Henrich et al., 2002). Also, problems such as low production levels and instability in certain environments need to be addressed (Abee et al., 1995). For example, bacteriocin expression systems in *Carnobacterium maltaromaticum* LV17C for colicin V (McCormick et al., 1999) and brochocin-C (McCormick et al., 1998) were developed using rolling circle plasmids. However, these plasmids were not stable in the absence of antibiotic pressure and would not be suitable for production of bacteriocins in foods. In addition, plasmids must be stable during storage at low temperatures if they are to be used as expression vectors for bacteriocins that will serve as biopreservatives in foods. It was observed that low temperatures cause a decrease in plasmid stability with a resulting loss of bacteriocin production (see Chapter 2). Currently there is no information in the literature on the effect of refrigeration temperature on stability of theta-type plasmids in LAB. Thus research is needed for the development of systems that are optimally suited for specific conditions or applications (Axelsson et al., 1998; Rodriguez et al., 2003).

The effect of low temperature on the stability of five theta-type replicating plasmids in *C. maltaromaticum* UAL26 was investigated. These plasmids were chosen because theta replicating plasmids do not produce single-stranded intermediates and are more stable than rolling circle plasmids (Kiewiet et al., 1993) and therefore, may be more desirable for use as vectors for bacteriocin genes. There may be a connection between the ability of the original host organisms of these derivative plasmids to grow at refrigeration temperatures and plasmid stability at low temperature. The development of efficient methods for heterologous expression systems is an important goal in bacteriocin research (Axelsson et al., 1998) and knowledge of the growth temperature characteristics of the original host organism could be useful in the selection of plasmids with an increased likelihood of stability. A plasmid with excellent stability at refrigeration temperature was studied further to determine whether gene products from the replication region are involved in plasmid stability at low temperature.

3.2 Materials and methods

3.2.1 *Bacterial strains and plasmids*

The bacterial strains and plasmids used in this study are listed in Table 3.1. Bacterial strains used for plasmid isolations were grown under the following conditions. *Carnobacterium* spp. were grown in All Purpose Tween (APT) broth (Difco Laboratories, Becton Dickinson Microbiology Systems, Sparks, Maryland) at 25°C. *Enterococcus faecalis* JH2-2, *Lactobacillus plantarum* caTC2R, and *Lactococcus lactis* LM0230 were grown in APT broth at 30°C. *E. coli* were grown in Brain Heart Infusion (BHI) broth (Difco) at 35°C. Agar plates were made by addition of 1.5% (wt vol^{-1}) agar (Difco) to broth media. Erythromycin (Sigma-Aldrich Canada Ltd., Oakville, Ontario) at concentrations of 5 μg ml^{-1} for LAB and 200 μg ml^{-1} for *E. coli* or chloramphenicol (Sigma) at a concentration of 5 μg ml^{-1} were added as selective agents when appropriate. Stock cultures of the bacterial strains were stored at -70°C in broth media containing 20% (vol vol^{-1}) glycerol.

Table 3.1. Bacterial strains and plasmids used in this study.

Bacterial Strain or Plasmid	**Relevant Characteristics**[a]	**Reference or Source**
Strains		
Carnobacterium maltaromaticum UAL26	host strain for transformed plasmids used in this study	Our collection
E. coli XL1 Blue pTRKH2	contains pTRKH2	O'Sullivan and Klaenhammer, 1993
Lactococcus lactis LM0230	contains pHW800	Wyckoff et al., 1996
Enterococcus faecalis JH2-2	contains pUCB820	Benachour et al., 1995
Carnobacterium divergens NCIMB 702855	contains pCD3.4	NCIMB[b]
Lactobacillus plantarum caTC2R	contains pCaT	Jewel and Collins-Thompson, 1989
Tetragenococcus halophilus ATCC 33315	contains pUCL287	Benachour et al., 1997
Enterococcus faecalis ATCC 14508	contains pAMβ1	Clewell et al., 1974
Plasmids		
pCD3.4	3.4 kb	Our collection
pCD11	derivative of pCD3.4, Em^{R}, 4.4 kb	Our collection
pCaT	Cm^{R}, 8.5 kb	Ahn et al., 1992
pUCB820	derivative of pUCL287, Em^{R}, 5.5 kb	Benachour et al., 1997
pHW800	derivative of pMBB1, Cm^{R}, 3.8 kb	Wyckoff et al., 1996
pTRKH2	derivative of pAMβ1, Em^{R},6.9 kb	O'Sullivan and Klaenhammer, 1993
pVBL	pTRKH2 containing a 1541bp fragment from pCD11	this study
pVBS	pTRKH2 containing a 623bp fragment from pCD3.4	this study

---

[a] Em^{R}, erythromycin resistant; Cm^{R}, chloramphenicol resistant

[b] NCIMB, National Collection of Industrial, Food and Marine Bacteria, Aberdeen, Scotland

3.2.2 *Determination of ability to grow at low temperature*

Cultures were inoculated into the appropriate broth media from frozen stocks and were subcultured at least once before initiation of the experiment. *C. divergens* NCIMB 702855 was grown in BHI broth (Difco) at 25°C for 24 h. *Ent. faecalis* ATCC 14508 and *Lb. plantarum* caTC2R were grown in BHI broth at 30°C for 24 h. *Tetragenococcus* *halophilus* ATCC 33315 was grown at 30°C for 48 h in Lactobacilli MRS broth (Difco) to which 5% NaCl and 0.5% glucose (MRSNG) were added. Fully grown (ca. log 9 cfu ml^{-1}) cultures were serially diluted in sterile 0.1% peptone water (Difco) and 100 μl of the 10^{-4} dilution was added to each of two tubes of 10 ml of BHI or MRSNG broth and incubated at 4 ± 0.5°C and 2 ± 0.5°C. At regular intervals during storage at 4 or 2°C, samples (100 μl) were removed from each tube, serially diluted in sterile 0.1% peptone water, plated onto either BHI or MRSNG agar, and colonies were counted after incubation at the appropriate temperature.

3.2.3 *DNA isolation and transformation*

Plasmid DNA from *E. coli* was isolated using the large scale plasmid extraction method described by Sambrook and Russell (2001). Plasmid DNA from LAB was isolated using a method described by Sambrook and Russell (2001) with the following modifications. Harvested and washed cells were incubated at 37°C for 30 min in 8 ml of Solution I containing 25% sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM NaCl, and 15 mg ml^{-1} lysozyme (Sigma) prior to alkali lysis using 3% SDS. Plasmid DNA was purified by density gradient ultracentrifugation in CsCl-ethidium bromide. Competent *C. maltaromaticum* UAL26 were prepared according to the method described by van Belkum and Stiles (1995) with the following modifications. Cells were grown in APT broth supplemented with 4% (wt vol^{-1}) glycine, harvested and the cells were washed three times with ice-cold electroporation buffer (0.5 M sucrose, 2.5 mM $CaCl_2$). Prior to transformation, 40 μl of cell suspension was mixed with 1 μl of plasmid DNA and held on ice for 15 min in a 0.2 cm electroporation cuvette. Transformation of *C. maltaromaticum* UAL26 with each of the 5 theta replicating plasmids (pCD11, pCaT, pUCB820, pHW800, pTRKH2) by electroporation was performed using a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, California) set at 100Ω and 2.5kV. Immediately after electroporation, 1 ml of APT broth containing 0.5 M sucrose and 20 mM $MgCl_2$ was added to the cells that were then incubated at 25°C for 3 h. After incubation, the cells were plated on APT agar containing the appropriate antibiotic and plates were examined for transformants after 3 to 4 days at 25°C.

3.2.4 *Segregational stability*

The segregational stability of the five theta replicating plasmids transformed into *C. maltaromaticum* UAL26 was determined over 100 generations. The percent segregational stability reported was the average of three experiments. Frozen stock cultures of *C. maltaromaticum* UAL26 previously transformed with each of the theta replicating plasmids were grown overnight at 25°C in APT broth supplemented with either erythromycin or chloramphenicol. The cultures were subcultured at least once more in antibiotic-containing broth prior to initiation of the stability studies. Fully grown cultures (ca. log 9 cfu ml^{-1}) were serially diluted using sterile 0.1% peptone water and 100 μl of the 10^{-4} dilution was inoculated into 10 ml of fresh APT broth without antibiotic and incubated at 4 ± 0.5°C and 25 ± 0.5°C until the cultures were fully grown (approximately 20 generations). These fully grown cultures were serially diluted in sterile 0.1% peptone water and 100 μl of the 10^{-4} dilution was inoculated into 10 ml of fresh APT broth and incubated at 4 ± 0.5°C and 25 ± 0.5°C to obtain another 20 generations of growth. This procedure was repeated until 100 generations of growth was obtained. After every 20 generations 100 μl of the 10^{-6} dilution from fully grown cultures was plated onto non-selective APT agar plates to obtain approximately 300 colonies per plate. The plates were incubated at 25 ± 0.5°C for 48 h. The percentage of erythromycin or chloramphenicol resistant clones was determined by picking 50 colonies onto an APT agar plate supplemented with erythromycin or chloramphenicol. The plates were incubated at 25 ± 0.5°C for 48 h and the number of colonies was enumerated and percent segregational stability was calculated.

3.2.5 *Cloning of replication regions*

Restriction endonucleases and T4 DNA ligase (Invitrogen Canada Inc., Burlington, Ontario) were used according to the supplier's instructions. Plasmid, pCD11, was initially digested with *Ava*II and then treated with Klenow large fragment polymerase (Gibco BRL, Gaithersburg, Maryland) to create blunt ends and was digested with *Mbo*I. The resulting 1541bp DNA fragment (Figure 3.1) containing 12 and 22 bp repeats (iterons), putative DnaA box and origin of replication, and the *repA* gene (van Belkum and Stiles, 1999) was cloned into the *Eco*RV and *Bam*HI sites of pTRKH2. The resulting plasmid, pVBL, was transformed into *C. maltaromaticum* UAL26.

A 951bp fragment of DNA, a shortened version of the 1541bp fragment, was amplified by PCR using RWO56 (5'-AAACGATACGATACACAAAGTA) and RWO57 (5'-AGATGAGATAAGGTCTACATGGA) as the primers with pCD3.4 sequence as a template. The PCR product was digested with *Mbo*I and *Sac*I and the resulting 623bp fragment, containing the 12 bp repeats, the putative DnaA box and origin of replication but lacking the DNA iterons and *repA* gene, (Figure 3.1) was cloned into the *Bam*HI and *Sac*I sites of pTRKH2. This derivative plasmid, pVBS, was transformed into *C. maltaromaticum* UAL26. The oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer (Applied Biosystems, Foster City, California). DNA was amplified in a 100 μl reaction mixture with a temperature cycler (OmniGene; InterSciences Inc., Markham, Ontario, Canada). PCR mixtures contained 1.0 μM of each primer, 200 μM deoxynucleotide triphosphates (Invitrogen Canada Inc.), 2.5 mM $MgCl_2$, 2.5 U of *Taq*Plus Precision polymerase (Stratagene Cloning Systems, LaJolla, CA.) and 1X *Taq*Plus Precision buffer (Stratagene). DNA was amplified with 30 cycles (denaturation, 94°C, 1 min; annealing, 58°C, 1min; extension, 72°C, 2 min) followed by a final extension step at 72°C for 10 min.

Segregational stability of pVBL and pVBS was determined as described above.

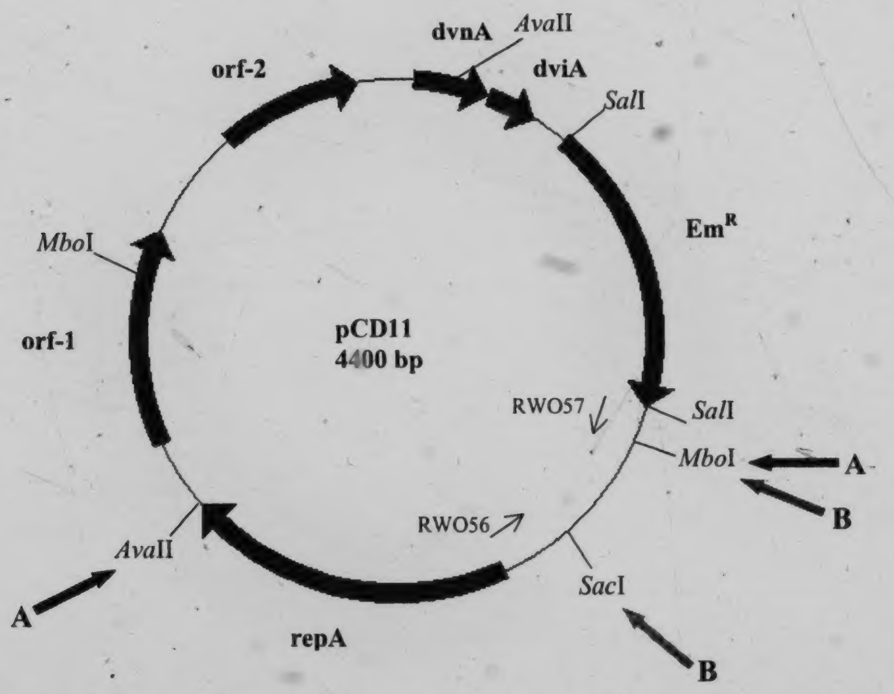

Figure 3.1. Plasmid map of pCD11 with relevant restriction sites and locations of primers used in the study.

Region between the large dark arrows (A) indicates the 1541 bp fragment that was inserted into pTRKH2 and resulted in pVBL. Region between the large light gray arrows (B) indicates the 623 bp fragment inserted into pTRKH2 and resulted in pVBS.

3.3 Results and discussion

The plasmids used in this study were selected based on their ability to replicate in several species of LAB and on their reported segregational stability in LAB. van Belkum and Stiles (1999) transformed other *C. maltaromaticum* strains, including *C. maltaromaticum* UAL26, with a derivative of pCD3.4, pCD11, and previously demonstrated stability in *C. maltaromaticum* UAL26. Ahn et al. (1992) transformed pCaT into *C. maltaromaticum* UAL26 as well as several other strains of LAB. The segregational stability of pUCB820, a derivative of pUCL287, was 77 and 54% after 25 and 100 generations, respectively, in *Ent. faecalis* JH2-2 and the host range of pUCL287 included the genera *Pediococcus, Enterococcus, Lactobacillus,* and *Leuconostoc* (Benachour et al., 1997). Wyckoff et al. (1996) reported that pHW800, a derivative of pMBB1, was maintained in *L. lactis* LM0230 by 100% of the population after 100 generations of growth and had been successfully transformed into *Lactococcus, Leuconostoc,* and *Pediococcus* hosts. Plasmid, pTRKH2, based on the pAMβ1replicon, had been transformed into *L. lactis, Ent. faecalis, Streptococcus thermophilus,* and *Lb. johnsonii* (O'Sullivan and Klaenhammer, 1993).

The ability of the original host strains *C. divergens* NCIMB 702855, *Ent. faecalis* ATCC 14508, *Lb. plantarum* caTC2R, and *T. halophilus* ATCC 33315 of the theta-type plasmids pCD11, pTRKH2, pCaT, and pUCB820, respectively, to grow in broth stored at 2 or 4°C was evaluated. *C. divergens* NCIMB 702855 and *Lb. plantarum* caTC2R both grew at the refrigeration temperatures (Figure 3.2). *C. divergens* NCIMB 702855 reached maximum population at 12 days and *Lb. plantarum* caTC2R reached maximum population at 16 days of incubation at 4°C. At 2°C, *C. divergens* NCIMB 702855 reached maximum population at 16 days of incubation and *Lb. plantarum* caTC2R reached maximum population after 28 days (data not shown). *Ent. faecalis* ATCC 14508 and *T. halophilus* ATCC 33315 did not grow at either 2°C (data not shown) or 4°C (Figure 3.2) and numbers of *Ent. faecalis* ATCC 14508 declined after 12 days incubation at 2°C (data not shown).

The theta replicating plasmids, pCD11, pCaT, pUCB820, pHW800, and pTRKH2, were transformed into *C. maltaromaticum* UAL26 and were evaluated for segregational stability at 25 and 4°C. Excellent stability was observed for pCD11 at both temperatures with nearly 100% of the strains retaining plasmids after 100 generations (Figure 3.3). These results were the same as those previously observed when pCD11 was transformed into three *Carnobacterium* spp. and tested for stability during incubation at 25 and 4°C in broth culture (see Chapter 2). Plasmid, pCaT, had similar segregational stability at both incubation temperatures (Figure 3.3). Plasmid, pUCB820, was very stable at 25°C (Figure 3.3.A); however, stability was dramatically reduced at 4°C (Figure 3.3B). Plasmid, pHW800, was more stable at 4°C than at 25°C (Figure 3.3). Plasmid, pTRKH2,

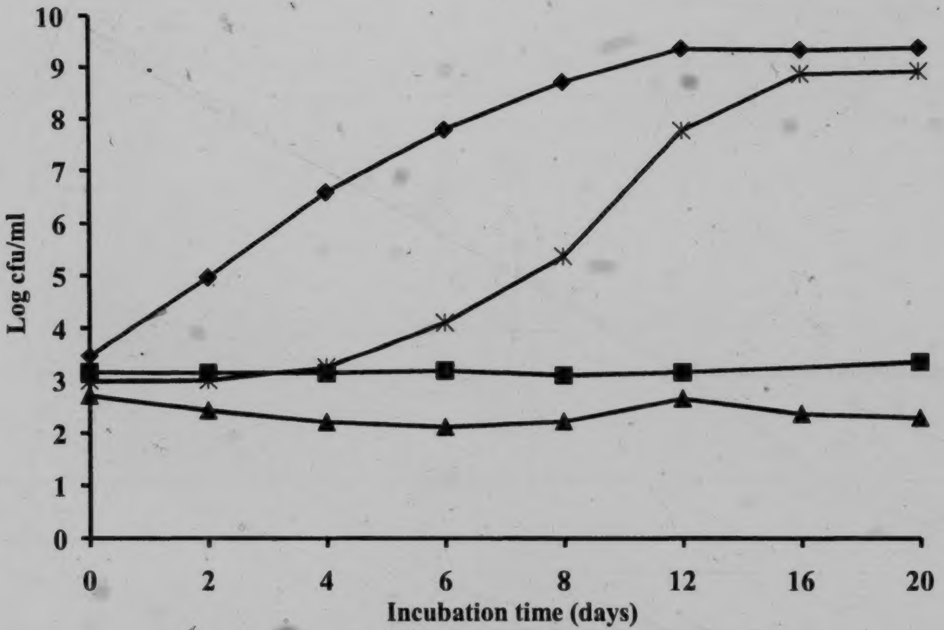

Figure 3.2. Growth of bacterial strains in broth media at 4°C.
◆, *C. divergens* NCIMB 702855; ✳, *Lb. plantarum* caTC2R; ■, *Ent. faecalis* ATCC 14508; ▲, *T. halophilus* ATCC 33315.

showed a gradual decline in plasmid stability at 25°C (Figure 3.3.A) and was not stable at 4°C (Figure 3.3.B) and in fact, *C. maltaromaticum* UAL 26 transformed with pTRKH2 was unable to grow at 4°C until the plasmid was lost (data not shown). The instability of pTRKH2 in *Carnobacterium* spp. had been previously observed during incubation at 4°C in broth (see Chapter 2). The decreased segregational stability of the plasmids at low temperature corresponded with the inability of the original host strains to grow at refrigeration temperatures. For example, both pTRKH2 and pUCB820 were highly unstable in *C. maltaromaticum* UAL26 at 4°C and they are derivatives of plasmids originally isolated from bacteria (*Ent. faecalis* ATCC 14508 and *T. halophilus* ATCC 33315) that did not grow at 2 or 4°C. In contrast, pCD11 showed excellent stability at

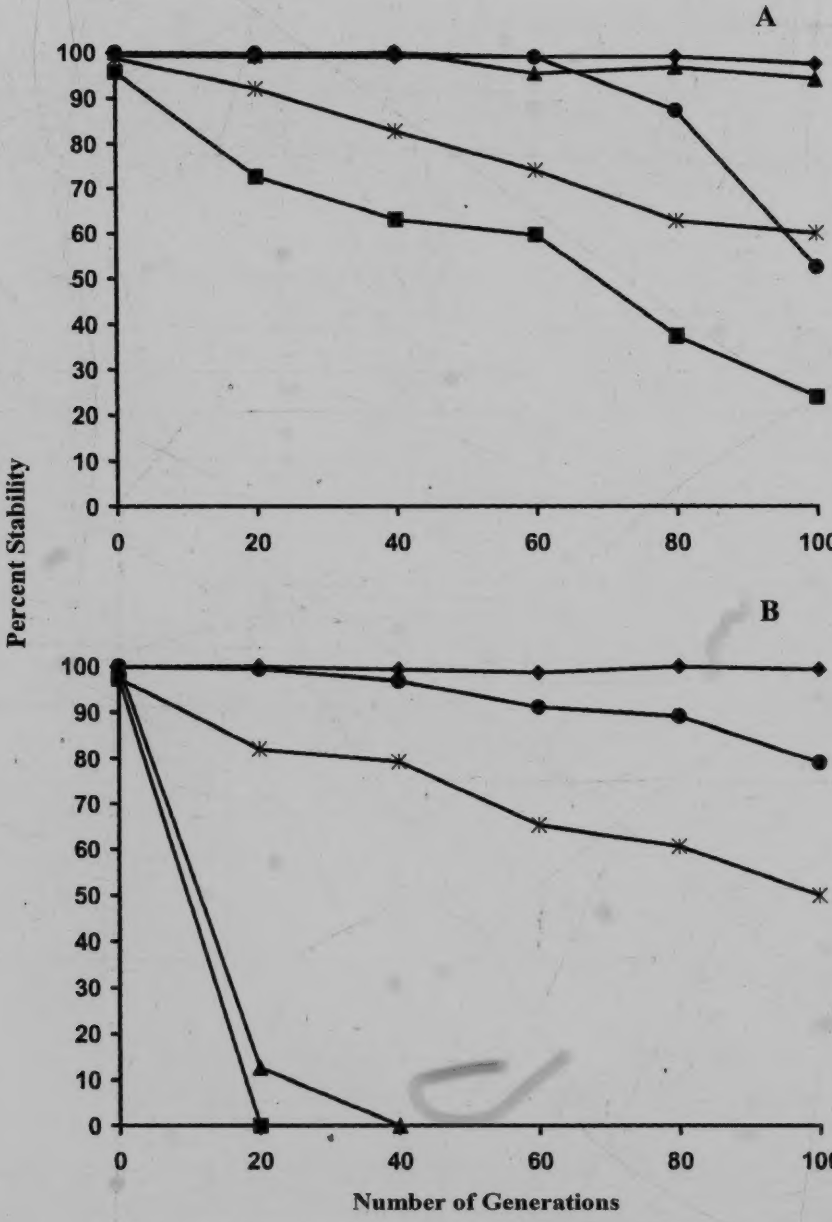

Figure 3.3. Stability of plasmids in *C. maltaromaticum* UAL26 grown in APT broth incubated at 25°C (A) and 4°C (B).

◆, pCD11; ✳, pCaT; ■, pTRKH2; ▲, pUCB820; ●, pHW800.

4°C and was a derivative of a plasmid isolated from *C. divergens* NCIMB 702855 that was able to grow at 2 and 4°C. Refrigeration temperature did not have a deleterious effect on the stability of pCaT or pHW800 in *C. maltaromaticum* UAL26. Plasmid, pCaT, was isolated from *Lb. plantarum* caTC2R that grew at 2 and 4°C. Plasmid, pHW800, a derivative of pMBB1, was isolated from *Ent. faecium* 226, a bacterium of unknown origin that was originally designated as a *Leuconostoc* species (Wyckoff et al., 1996). Although it could not be determined if *Ent. faecium* 226 could grow in broth stored at 2 and 4°C because the strain was unavailable, other strains of *Ent. faecium* have been isolated from refrigerated foods such as raw and processed meats, as well as in fermented meat and dairy products (Vancanneyt et al., 2002) so it is possible that *Ent. faecium* 226 would be able to grow at refrigeration temperatures.

Although no information in the literature on the effects of low temperatures on plasmid stability could be found, other researchers have noted temperature sensitivity of theta plasmids in LAB incubated at high temperatures. Horng et al. (1991) showed that maintenance of pSK11L was temperature sensitive in *L. lactis* when the cultures were tested during incubation at 25, 32, 37 and 39°C. They found that there was no significant difference between loss frequencies at 25 and 32°C but plasmid loss rates increased an average of 6-fold at 37°C and at least 10-fold at 39°C (Horng et al., 1991).

Researchers have shown that the replication region is involved in reduced plasmid stability at high temperatures (Duan et al., 1999; Frere et al., 1998; Horng et al., 1991). To determine if the replication region of pCD11, a plasmid that had excellent stability at 4°C, was involved in plasmid stability at low temperatures, two fragments were inserted into the plasmid, pTRKH2, which was not stable at low temperature. The 1541bp *Ava*II-*Mbo*I fragment contained 12 and 22bp repeats (iterons), putative DnaA box and origin of replication, and the *repA* gene from pCD11 (van Belkum and Stiles, 1999). When this fragment was inserted into pTRKH2, the derivative plasmid, pVBL, had excellent segregational stability (100%) in *C. maltaromaticum* UAL26 for 100 generations during incubation at 25 and 4°C (Figure 3.4). Insertion of the 623bp *Mbo*I-*Sac*I fragment, a shortened version lacking the 22 bp DNA iterons and the *repA* gene but containing a

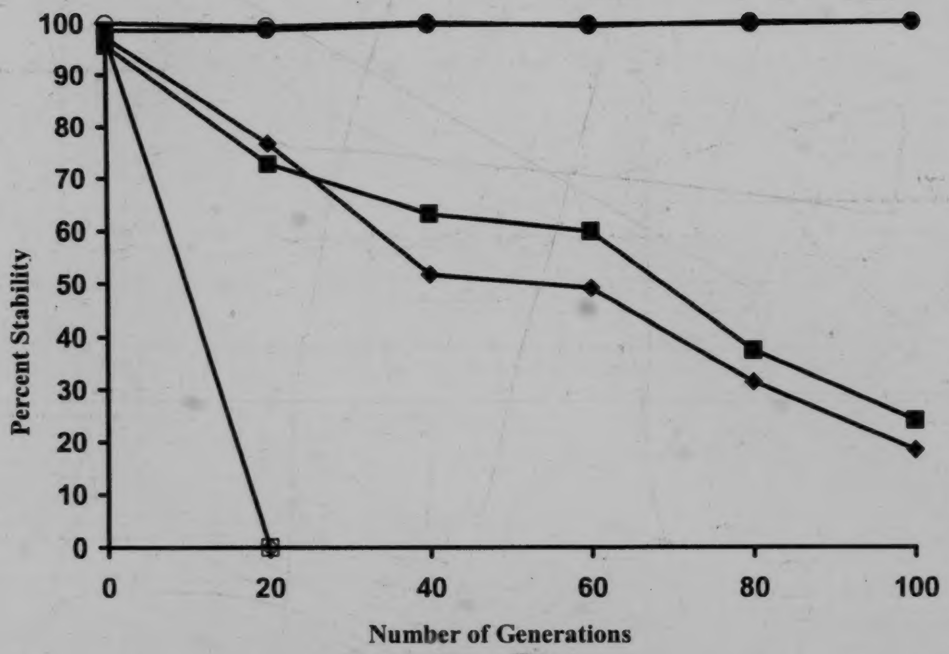

Figure 3.4. Stability of ■, pTRKH2; ●, pVBL; ◆, pVBS in *C. maltaromaticum* UAL26 grown in APT broth incubated at 25°C (closed symbols) and 4°C (open symbols).

region affecting segregational stability and replication (van Belkum and Stiles, 1999), into pTRKH2 caused a slight decline in the stability of pVBS at 25°C but did not improve stability at 4°C (Figure 3.4). The dramatic increase in the stability of pVBL at 4°C indicated that the whole replication region, including the *repA* gene and 22 bp DNA iterons, plays an important role in stability at refrigeration temperature. Duan et al. (1999) identified a thermosensitive replication region from the thermostable plasmid, pND324, and demonstrated that a non-coding *cis*-acting element in addition to the minimal replication region was essential for stability at higher temperature (37°C). In this study we did not determine if just the minimal replication region of pCD11, which includes the replication initiation site, DNA iterons, and the *repA* gene, is sufficient to ensure plasmid stability at 4°C or whether additional regions of the plasmid are essential.

Therefore, future experiments using deletion derivatives need be conducted to more accurately identify the regions responsible for the stable maintenance of pCD11 at 4°C.

The development and optimization of LAB and bacteriocins as biopreservatives to control undesirable bacteria is a primary focus of many laboratories involved in research related to food safety and quality (Luchansky, 1999) and it is important to develop safe, stable and effective genetic modification and expression tools (Konings et al., 2000). Ensuring stable production can depend on choosing appropriate plasmid vectors for bacteriocin genes. Results of this study indicate that incubation temperature of the host cells can have a dramatic effect on the stability of some theta replicating plasmids. Knowing whether the bacterial strain from which the plasmid originated has the ability to grow at refrigeration temperature may assist in selecting a plasmid with increased likelihood of stability in a heterologous host during extended storage at low temperatures. The functions of the replication region and its effect on plasmid stability at low temperature need further investigation. To our knowledge, published studies on plasmid stability and temperature have involved ambient or higher temperatures. This is the first study that we are aware of that has investigated the effect of low temperatures on the stability of theta plasmids in LAB.

3.4 References

Abee, T., Krockel, L., and Hill, C. (1995) Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. *Int J Food Microbiol* **28**: 169-185.

Ahn, C., Collins-Thompson, D., Duncan, C., and Stiles, M.E. (1992) Mobilization and location of the genetic determinant of chloramphenicol resistance from *Lactobacillus plantarum* caTC2R. *Plasmid* **27**: 169-176.

Arnau, J., Hjerl-Hansen, E., and Israelsen, H. (1997) Heterologous gene expression of bovine plasmin in *Lactococcus lactis*. *Appl Microbiol Biotechnol* **48**: 331-338.

Axelsson, L., Katla, T., Bjornslett, M., Eijsink, V.G., and Holck, A. (1998) A system for heterologous expression of bacteriocins in *Lactobacillus sake*. *FEMS Microbiol Lett* **168**: 137-143.

Benachour, A., Frere, J., Flahaut, S., Novel, G., and Auffray, Y. (1997) Molecular analysis of the replication region of the theta-replicating plasmid pUCL287 from *Tetragenococcus (Pediococcus) halophilus* ATCC33315. *Mol Gen Genet* **255**: 504-513.

Bernasconi, E., Germond, J.E., Delley, M., Fritsche, R., and Corthesy, B. (2002) *Lactobacillus bulgaricus* proteinase expressed in *Lactococcus lactis* is a powerful carrier for cell wall-associated and secreted bovine beta-lactoglobulin fusion proteins. *Appl Environ Microbiol* **68**: 2917-2923.

Clewell, D.B., Yagi, Y., Dunny, G.M., and Schultz, S.K. (1974) Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. *J Bacteriol* **117**: 283-289.

Duan, K., Liu, C.Q., Liu, Y.J., Ren, J., and Dunn, N.W. (1999) Nucleotide sequence and thermostability of pND324, a 3.6-kb plasmid from *Lactococcus lactis*. *Appl Microbiol Biotechnol* **53**: 36-42.

Frere, J., Benachour, A., Giard, J.C., Laplace, J.M., Flahaut, S., and Auffray, Y. (1998) A new theta-type thermosensitive replicon from *Lactococcus lactis* as an integration vector for *Enterococcus faecalis*. *FEMS Microbiol Lett* **161**: 107-114.

Henrich, B., Klein, J.R., Weber, B., Delorme, C., Renault, P., and Wegmann, U. (2002) Food-grade delivery system for controlled gene expression in *Lactococcus lactis*. *Appl Environ Microbiol* **68**: 5429-5436.

Horn, N., Martinez, M.I., Martinez, J.M., Hernandez, P.E., Gasson, M.J., Rodriguez, J.M., and Dodd, H.M. (1998) Production of pediocin PA-1 by *Lactococcus lactis* using the lactococcin A secretory apparatus. *Appl Environ Microbiol* **64**: 818-823.

Horng, J.S., Polzin, K.M., and McKay, L.L. (1991) Replication and temperature-sensitive maintenance functions of lactose plasmid pSK11L from *Lactococcus lactis* subsp. *cremoris*. *J Bacteriol* **173**: 7573-7581.

Jewell, B., and Collins-Thompson, D.L. (1989) Characterization of chloramphenicol resistance in *Lactobacillus plantarum* caTC2. *Curr Microbiol* **19**: 343-346.

Kiewiet, R., Kok, J., Seegers, J., Venema, G., and Bron, S. (1993) The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **59**: 358-364.

Konings, W.N., Kok, J., Kuipers, O.P., and Poolman, B. (2000) Lactic acid bacteria: the bugs of the new millennium. *Curr Opin Microbiol* **3**: 276-282.

Langella, P., and Le Loir, Y. (1999) Heterologous protein secretion in *Lactococcus lactis*: a novel antigen delivery system. *Braz J Med Biol Res* **32**: 191-198.

Luchansky, J.B. (1999) Overview on applications for bacteriocin-producing lactic acid bacteria. *Antonie Van Leeuwenhoek* **76**: 335.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E., and Van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. *Appl Environ Microbiol* **64**: 4757-4766.

McCormick, J.K., Klaenhammer, T.R., and Stiles, M.E. (1999) Colicin V can be produced by lactic acid bacteria. *Lett Appl Microbiol* **29**: 37-41.

O'Sullivan, D.J., and Klaenhammer, T.R. (1993) High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene* **137**: 227-231.

Ribeiro, L.A., Azevedo, V., Le Loir, Y., Oliveira, S.C., Dieye, Y., Piard, J.C., Gruss, A., and Langella, P. (2002) Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microbiol* **68**: 910-916.

Rodriguez, J.M., Martinez, M.I., Horn, N., and Dodd, H.M. (2003) Heterologous production of bacteriocins by lactic acid bacteria. *Int J Food Microbiol* **80**: 101-116.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Seegers, J.F. (2002) Lactobacilli as live vaccine delivery vectors: progress and prospects. *Trends Biotechnol* **20**: 508-515.

van Belkum, M.J., and Stiles, M.E. (1995) Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. *Appl Environ Microbiol* **61**: 3573-3579.

van Belkum, M.J., and Stiles, M.E. (1999) Characterization of a theta-type plasmid from *Carnobacterium divergens* and its use as a bacteriocin expression vector. In *Sixth Symposium on Lactic Acid Bacteria: Genetics, Metabolism and Applications* Veldhoven, the Netherlands: Federation of European Microbiological Societies and Netherlands Society for Microbiology.

Vancanneyt, M., Lombardi, A., Andrighetto, C., Knijff, E., Torriani, S., Bjorkroth, K.J., Franz, C.M., Foulquie Moreno, M.R., Revets, H., De Vuyst, L., Swings, J., Kersters, K., Dellaglio, F., and Holzapfel, W.H. (2002) Intraspecies genomic groups in *Enterococcus faecium* and their correlation with origin and pathogenicity. *Appl Environ Microbiol* **68**: 1381-1391.

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C., and Stiles, M.E. (1995) A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. *J Bacteriol* **177**: 3143-3149.

Wyckoff, H.A., Barnes, M., Gillies, K.O., and Sandine, W.E. (1996) Characterization and sequence analysis of a stable cryptic plasmid from *Enterococcus faecium* 226 and development of a stable cloning vector. *Appl Environ Microbiol* **62**: 1481-1486.

4. Influence of lactic acid bacteria promoters on brochocin-C production by heterologous *Carnobacterium maltaromaticum*

4.1 Introduction

Bacteriocins and bacteriocin-producing lactic acid bacteria (LAB) have the potential to serve as biopreservatives to enhance safety and increase the storage life of foods. Bacteriocins are antimicrobial peptides or proteins produced by bacteria that have been shown to be effective for inhibiting spoilage or pathogenic microorganisms in food systems (Leisner et al., 1996; Luchansky et al., 1992; Morgan et al., 2001; Muriana, 1996; Scannell et al., 2000a; Scannell et al., 2000b; Schillinger et al., 1991). A bacteriocin with promising potential for biopreservation is brochocin-C, which has a broad activity spectrum. Brochocin-C is produced by *Brochothrix campestris* ATCC 43754 and was originally reported by Siragusa and Cutter (Siragusa and Cutter, 1993) and was later characterized by McCormick et al. (McCormick *et al.*, 1998). It would be desirable to have brochocin-C heterologously produced by LAB such as *Carnobacterium maltaromaticum* because the wild-type producer was isolated from soil and grass (Talon et al., 1988) and would not be considered a food-grade organism since *B. campestris* is not normally associated with food and because purification of the bacteriocin is a laborious process (McCormick et al., 1998). Brochocin-C has been successfully produced by *Carnobacterium* spp. using a plasmid vector that is stable in the absence of antibiotic pressure and stable during extended storage at refrigeration temperatures (see Chapter 2). Transcription of the brochocin-C genes, including the signal peptide from divergicin A, was under the control of the P32 promoter. One of the potential drawbacks associated with the use of this constitutive promoter was the dramatic decrease in brochocin-C production from *Carnobacterium* spp. compared to production by the wild-type producer. Heterologous expression of brochocin-C resulted in a 4- to 8-fold decrease in bacteriocin activity detected with the spot-on-lawn assay (see Chapter 2).

The focus of this study was to determine the effect of several constitutive promoters from LAB on brochocin-C production in *C. maltaromaticum* LV17C to determine whether heterologous production of brochocin-C could be enhanced. Five sequenced and characterized constitutive promoters of varying strengths originally isolated from *Lactococcus lactis* subsp. *cremoris* Wg2 chromosomal DNA (van der Vossen et al., 1987), promoter regions upstream of other bacteriocin genes, an uncharacterized promoter region controlling expression of a chloramphenicol acetyltransferase gene, as well as uncharacterized native promoter fragments from *C. maltaromaticum* LV17C and *C. maltaromaticum* UAL26 chromosomal DNA were tested for activity (strength) in *C. maltaromaticum* LV17C. Brochocin-C activity levels were determined when transcription of the bacteriocin genes including the signal peptide from divergicin A was under the control of these various constitutive promoters.

4.2 Materials and methods

4.2.1 *Bacterial strains and plasmids*

The bacterial strains and plasmids used in this study are listed in Table 4.1. Lactic acid bacteria and *Brochothrix campestris* 43754 were grown in All Purpose Tween (APT) broth (Difco Laboratories, Becton Dickinson Microbiology Systems, Sparks, Maryland) at 25°C. *E. coli* were grown in Brain Heart Infusion (BHI) broth (Difco) at 35°C. Agar plates were made by addition of 1.5% (wt vol^{-1}) agar (Difco) to broth media. Soft agar was made by the addition of 0.75% (wt vol^{-1}) agar to broth media. Erythromycin (Sigma-Aldrich Canada Ltd., Oakville, Ontario) at a concentration of 5 µg ml^{-1} for LAB and 200 µg ml^{-1} for *E. coli* or chloramphenicol (Sigma) at concentrations of 5 to 60 µg ml^{-1} were added as selective agents when appropriate. Stock cultures of the bacterial strains were stored at -70°C in broth media containing 20% (vol vol^{-1}) glycerol.

4.2.2 *DNA isolation and transformation*

Plasmid DNA (pGKV210, pGKV221, pGKV223, pGKV232, pGKV244, and pGKV259) from *E. coli* strains was isolated using the small scale plasmid extraction method described by Sambrook and Russell (2001). Plasmid DNA from LAB was also

Table 4.1. Bacterial strains and plasmids used in this study.

Bacterial Strain or Plasmid	**Relevant Characteristics**[a]	**Reference or Source**
Strains		
Carnobacterium maltaromaticum LV17C	plasmid free	Ahn and Stiles, 1990a
E. coli BHB2600	strains containing pGKV210, pGKV221, pGKV223, pGKV232, pGKV244, and pGKV259	van der Vossen et al., 1987
C. maltaromaticum UAL26	produces piscicolin 126, bacteriocin genes on chromosome	Our collection
Lactobacillus plantarum caTC2R	contains pCaT which has *cat* (chloramphenicol acetyltransferase) gene	Ahn et al., 1992
Leuconostoc gelidum UAL187	produces leucocin A, bacteriocin genes on plasmid	Hastings and Stiles, 1991
Brochothrix campestris ATCC 43754	produces brochocin-C, bacteriocin genes on chromosome	Siragusa and Cutter, 1993
C. maltaromaticum LV17A	produces carnobacteriocin A, bacteriocin genes on plasmid	Worobo et al., 1994
Plasmids		
pVB2-4	contains genes for Brc-C including signal peptide from divergicin A in front of b*rcA* and b*rcB*; b*rcI*; Em^R	Our collection
pCaT	*cat* gene, Cm^R, 8.5 kb	Ahn et al., 1992
pGKV210	promoter screening vector, *cat*-86 gene from *Bacillus pumilus*, Em^R, 4.4kb	van der Vossen et al., 1985
pGKV221	derivative of pGKV210, contains P21 promoter, *cat*-86 gene, Em^R	van der Vossen et al., 1987
pGKV223	derivative of pGKV210, contains P23 promoter, *cat*-86 gene, Em^R	van der Vossen et al., 1987
pGKV232	derivative of pGKV210, contains P32 promoter, *cat*-86 gene, Em^R	van der Vossen et al., 1987
pGKV244	derivative of pGKV210, contains P44 promoter, *cat*-86 gene, Em^R	van der Vossen et al., 1987
pGKV259	derivative of pGKV210, contains P59 promoter, *cat*-86 gene, Em^R	van der Vossen et al., 1987

[a] Em^R, erythromycin resistant; Cm^R, chloramphenicol resistant; Brc-C, brochocin-C; b*rcA*, brochocin A gene ; b*rcB*, brochocin B gene; b*rcI*, brochocin-C immunity gene

isolated using the above method with the following modifications. Harvested and washed cells were incubated at 37°C for 60 min in Solution I containing 15 mg ml^{-1} lysozyme (Sigma). Lysis Solution II was added and followed by the addition of 150 µl of 3 M potassium acetate. DNA was precipitated overnight at -20°C in 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. The DNA was washed twice with 70% ethanol, dried under vacuum at 45°C (Centrivap Console, Labconco Corporation, Kansas City, Missouri), and resuspended in TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)] with 0.1 mg ml^{-1} RNase. The tubes were incubated at 37°C for 30 min and the plasmid DNA was stored at 4°C.

Chromosomal DNA was isolated from *B. campestris* 43754 using a method adapted from Ausubel et al. (1995). Five milliliters of a fully grown culture (ca. log 9 cfu ml^{-1}) was centrifuged at 1,000 x *g* for 20 min at 4°C and the supernatant discarded. The pellet was resuspended in 580 µl of TE buffer with 67.5 µl of 10 mg ml^{-1} lysozyme. After incubating the resuspended cells at 37°C for 30 min, 40.5 µl of 10% SDS was added, the tube was gently mixed, and 4.1 µl of 20 mg ml^{-1} proteinase K was added. The tube was mixed and incubated at 37°C for 60 min. Seven hundred microliters of a mixture of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the mixture vortexed for 30 sec. The sample was centrifuged at 12,000 x *g* for 15 min at 4°C and the aqueous layer was removed to a new tube. The DNA was precipitated overnight at -20°C with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. The chromosomal DNA was washed with 70% ethanol and dried under vacuum at 45°C. The dried pellet was resuspended in 50 µl TE buffer with 0.1 mg ml^{-1} RNase, incubated at 37°C for 30 min and stored at 4°C.

Chromosomal DNA was isolated from *Carnobacterium* spp. using the above method as well as two additional methods. Method A: A single colony picked from an APT agar plate was inoculated into APT broth and incubated at 25°C overnight. One hundred and twenty five microliters of the fully grown culture (ca. log 9 cfu ml^{-1}) was streaked onto an APT agar plate and incubated at 25°C overnight. Using a sterile loop, the bacteria from the entire surface of the agar plate was removed and placed into a microfuge tube containing 1 ml of sterile, de-ionized purified water. The cells were centrifuged at 12,000 x *g* for 3 min and the supernatant discarded. The cells were washed in 1 ml TN 150 buffer [10 mM Tris (pH 8.0), 150 mM NaCl] and then resuspended in 1 ml TN 150 buffer. Eight hundred microliters of the cell suspension was transferred to a screwcap microfuge tube containing 0.5 ml dimethyldichlorosilane (DMCS) treated glass beads (100/120 mesh, Alltech Associates Inc. Deerfield, Illinois). The tube was shaken for 45 sec on setting 5.5 on a FastPrep instrument (FP120, Savant Instruments Inc., Holbrook, New York) and immediately placed on ice. After incubation on ice for 30 min, 500 µl of the supernatant was transferred to a new tube containing 500 µl of a mixture of phenol/chloroform/isoamyl alcohol (25:24:1). The mixture was vortexed and centrifuged at 12,000 x *g* for 6 min and the aqueous layer was removed. This extraction step was repeated once more and the chromosomal DNA in the aqueous layer was precipitated overnight at -20°C with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. The precipitated DNA was washed twice with 70% ethanol, dried under vacuum at 45°C, resuspended in 500 µl TE buffer and 0.1 mg ml^{-1} RNase, and incubated at 37°C for 60 min. Five hundred microliters of phenol/chloroform/isoamyl alcohol was added to the tube, centrifuged for 6 min at 12,000 x *g* and the aqueous layer removed to a new tube. The DNA was precipitated overnight at -20°C in sodium acetate and ethanol, washed twice with 70% ethanol, dried under vacuum at 45°C, resuspended in 20 µl TE buffer and was stored at 4°C.

Method B used the Magazorb DNA Mini-Prep kit (Cortex Biochem, San Leandro, California) according to the manufacturer's directions with the following modifications. Bacterial growth from the entire surface of an APT agar plate was removed with a sterile loop and resuspended in 1 ml TN 150 buffer. Two hundred microliters of the cell suspension was added to 300 µl of 12 mM Tris buffer (pH 8.0) in a screw cap tube containing DMCS treated glass beads. The tubes were shaken for 45 sec on setting 5.5 on a FastPrep instrument (Savant Instruments Inc.) and then immediately placed on ice. The cooled cell suspension was centrifuged at 1,000 x *g* for 5 min. The DNA was extracted according to the kit protocol and precipitated overnight at -20°C in 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. The precipitated DNA was washed twice with 70% ethanol, dried under vacuum at 45°C, resuspended in 20 μl TE buffer with 0.1 mg ml^{-1} RNase, incubated at 37°C for 60 min and stored at 4°C.

Competent *C. maltaromaticum* LV17C were prepared according to the method described by van Belkum and Stiles (1995) with the following modifications. Cells were grown in APT broth supplemented with 4% (wt vol^{-1}) glycine, harvested and the cells were washed three times with ice-cold electroporation buffer (0.5 M sucrose, 2.5 mM $CaCl_2$). Prior to transformation, 40 μl of cell suspension was mixed with 1 μl of plasmid DNA and held on ice for 15 min in a 0.2 cm electroporation cuvette. Transformation was performed using a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, California) set at 100 Ω and 2.5 kV. Immediately after electroporation, 1 ml of APT broth containing 0.5 M sucrose and 20 mM $MgCl_2$ was added to the cells that were then incubated at 25°C for 3 h. After incubation, the cells were plated on APT agar containing the appropriate antibiotic and plates were examined for transformants after 3 to 4 days at 25°C.

4.2.3 *Oligonucleotide synthesis and amplification reactions*

The sequences and description of the oligonucleotides used in this study are listed in Table 4.2. The oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer (Applied Biosystems, Foster City, California). The genes for brochocin-C including the signal peptide from divergicin A were amplified to include *Sal*I restriction sites at either end of the construct in a 50 μl reaction mixture using a DNA Engine® Peltier Thermal Cycler (PTC-200, MJ Research Inc., Waltham, Massachusetts) with 32 cycles (denaturation, 94°C, 1 min, annealing, 54°C, 1 min, extension, 72°C, 2 min) followed by a final extension step at 72°C for 10 min. The reaction mixture contained 1X *Taq* High Fidelity buffer, 2.5 mM $MgSO_4$, 200 μM deoxynucleotide triphosphates (Invitrogen Canada Inc., Burlington, Ontario), 1 U Platinum® *Taq* DNA polymerase High Fidelity (Invitrogen Canada Inc) and 1 μM of each primer. The promoter regions were amplified to include an *Eco*RI site on the 5' end

Table 4.2. Oligonucleotides used in this study.

Primer name	Template	Restriction sites added	Sequence (5' – 3')
Val-34	dvn::*brcA* dvn::*brcB brcI*	*Sal*I to start of signal peptide	CGCGGTCGACTGGAGGTTGGTATATATGAAA
Val-2	dvn::*brcA* dvn::*brcB brcI*	*Sal*I to end of brochocin-C immunity gene	TATAGTCGACGGTACCTTTGTACTAGTTAGAGA
Val-35	leucocin A promoter region	*Eco*RI to start of promoter region 70 nucleotides upstream of –35 region	GCGCGAATTCTTAAAAGTGAAATAATTAACAAATA
Val-36	leucocin A promoter region	*Bam*HI to end of promoter region 16 nucleotides downstream of –10 region	GCGCGGATCCATTTAATTCACATTCAATTATATAT
Val-37	carnobacteriocin A promoter region	*Eco*RI to start of promoter region 81 nucleotides upstream of –35 region	GCGCGAATTCGCACCCCCAATCACTTTC
Val-38	carnobacteriocin A promoter region	*Bam*HI to end of promoter region 18 nucleotides downstream of –10 region	GCGCGGATCCTATTTGAGTTACAAGAACATTC
Val-39	brochocin-C promoter region	*Eco*RI site to start of promoter region 73 nucleotides upstream of –35 region	GCGCGAATTCTATAAGAAACTATTGATAATACTA
Val-40	brochocin-C promoter region	*Bam*HI to end of promoter region 25 nucleotides downstream of –10 region	GCGCGGATCCTTACATACAAAAGATAGCATGA
Val-41	*cat* promoter region from pCaT	*Eco*RI site to start of promoter region	GCGCGAATTCTTGAGCGATTACTACGCAAT
Val-42	*cat* promoter region from pCaT	*Bam*HI to end of promoter region	GCGCGGATCCTAAATTTCATCTTATCTCTTTGT

and a *Bam*HI site on the 3' end using the above protocol except for an extension step of 72°C for 1 min.

4.2.4 *Isolation and cloning of LAB promoters*

Plasmids containing the *Lactococcus lactis* subsp. *cremoris* promoters (pGKV221, pGKV223, pGKV232, pGKV244, and pGKV259) were transformed into *C. maltaromaticum* LV17C.

Restriction endonucleases (Invitrogen Canada Inc.) and T4 DNA ligase (Invitrogen Canada Inc.) were used according to the supplier's instructions. Amplified promoter-containing regions from *B. campestris* ATCC 43754 (chromosomal DNA), *Lb. plantarum* caTC2R (plasmid DNA), and *Lc. gelidum* UAL187 (plasmid DNA) were digested with *Eco*RI and *Bam*HI restriction endonucleases. The promoter-screening vector, pGKV210, was also digested with these enzymes. The promoter-containing fragments were ligated into pGKV210 and the plasmids transformed into competent *C. maltaromaticum* LV17C. Transformants were screened for resistance to chloramphenicol and PCR amplification was performed to verify the presence of the promoter-containing fragments.

To isolate native promoters, chromosomal DNA from *C. maltaromaticum* LV17C and *C. maltaromaticum* UAL26 was digested with *Mbo*I and the promoter-screening vector was digested with *Bam*HI. The digests underwent ligation and the ligation mixtures were transformed into competent *C. maltaromaticum* LV17C. Transformants were isolated on plates containing erythromycin and then were screened for resistance to chloramphenicol.

4.2.5 *Promoter activity in C. maltaromaticum LV17C*

Promoter activity was determined by screening transformants for resistance to increasing concentrations of chloramphenicol. The transformants were grown 25°C for 24 h in APT broth containing erythromycin. The fully grown cultures (ca. log 9 cfu ml^{-1}) were serially diluted in sterile 0.1% peptone water (Difco). One hundred microliters of the 10^{-6} dilution of the fully grown culture was spread onto APT agar plates containing chloramphenicol that increased by 5 μg ml^{-1} intervals up to 60 μg ml^{-1}. The plates were incubated at 25°C for 48 h and the number of colony forming units counted. The breakpoint of resistance was arbitrarily assigned as the concentration of chloramphenicol where colony size became substantially reduced compared to the colonies on APT agar plates containing no chloramphenicol or when the number of colony forming units decreased by more than 1 log cfu ml^{-1}.

4.2.6 *Brochocin-C production in C. maltaromaticum LV17C*

To determine the effect of promoters, which demonstrated various degrees of chloramphenicol resistance, on brochocin-C production in *C. maltaromaticum* LV17C, the amplified brochocin-C construct, containing the divergicin A signal peptide fused in front of the *brcA* and *brcB* structural genes and the immunity gene, was digested with *Sal*I restriction endonuclease. The promoter-containing plasmids were also digested with *Sal*I, a restriction site immediately downstream of the promoters in pGKV210. The digested brochocin-C amplicon was ligated into the promoter-containing plasmids and the ligation mixtures were transformed into *C. maltaromaticum* LV17C. Transformants that grew on APT agar plates containing erythromycin were screened for brochocin-C production using the deferred inhibition assay (Ahn and Stiles, 1990a) with *C. maltaromaticum* LV17C containing pGKV210 used as the indicator strain. The amount of detectable brochocin-C activity produced by the various *C. maltaromaticum* LV17 strains was determined using the spot-on-lawn method (Ahn and Stiles, 1990b). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution yielding a clear zone of inhibition on the indicator lawn and was multiplied by a factor of 100 to obtain the AU ml^{-1} of the original supernatant (Franz et al., 1996). When a clear inhibition zone was followed by a turbid zone, the AU was taken to be the average of the final two dilutions (O'Keeffe et al., 1999).

4.3 Results and discussion

Constitutive promoters from LAB have successfully been used to increase the expression of heterologous genes (Kahala and Palva, 1999; Leenhouts et al., 1998; Piard et al., 1997). Previously P32, a constitutive promoter from *Lactococcus lactis* subsp. *cremoris* Wg2 (van der Vossen et al., 1987), had been used for the heterologous expression of brochocin-C genes in *Carnobacterium* spp. (McCormick *et al.*, 1998). However, there was substantially less detectable activity compared to production by *B. campestris* ATCC 43754, the wild-type producer (see Chapter 2). In this study several additional promoters were used in the genetic construct in an attempt to increase the heterologous production of brochocin-C by *C. maltaromaticum* LV17C.

Plasmids containing the various lactococcal promoters were transformed into *C. maltaromaticum* LV17C and transformants were screened for resistance to chloramphenicol to determine whether other *L. lactis* subsp. *cremoris* Wg2 promoters in addition to P32 (van der Vossen et al., 1987) were active in *Carnobacterium* spp. The nucleotide sequences of the lactococcal promoters are shown in Table 4.3. It was determined that four out of the five promoters resulted in high levels of resistance to chloramphenicol (Table 4.4). The promoter, P59 from pGKV259, showed very low activity (< 5 $\mu g\ ml^{-1}$ chloramphenicol resistance) in *C. maltaromaticum* LV17C even though the sequence of the –35 region matched the consensus sequence and the –10 region matched the consensus sequence for 5 out of 6 nucleotides (Table 4.3). This promoter was not used in subsequent cloning experiments to enhance the production of brochocin-C because of its low activity in *C. maltaromaticum* LV17C. The strength of the lactococcal promoters was different in *C. maltaromaticum* LV17C compared to the strength that was reported for *L. lactis* IL1403 (van der Vossen et al., 1987). In *L. lactis* IL1403 P59 was the strongest promoter followed by P23 then P21 and with P32 and P44 giving resistance to the lowest concentration of chloramphenicol (van der Vossen et al., 1987). In *C. maltaromaticum* LV17C, the strongest promoter was P23 followed by P21 then P32 and P44 and lastly P59, which produced no observable resistance to chloramphenicol. The strength of promoters is dependent on the context in which they are tested (Solem and Jensen, 2002) and may vary depending on the gene being expressed and the host organism used. The ranking of individual promoter strength changes depending on the organism tested as was evident in this study since the same promoter-containing vectors were used but very different promoter activities were observed with *C. maltaromaticum* LV17C as compared to that reported in *L. lactis.*

Table 4.3. Nucleotide sequences of lactococcal and bacteriocin promoter regions. The –35 regions are shown in bold and –10 regions are underlined. The consensus sequences for the –35 and –10 regions are shown with nucleotides represented by N.

Promoter	Nucleotide Sequence
Lactococcal P21	TTTC**TTGACA**GAAGAAGGCGAAAAATGGTATTATATTTAGGTAC
Lactococcal P23	ACTG**ATGACA**AAAAGAGCAAATTTTGATAAAATAGTATTAGAA
Lactococcal P32	GGAC**TAGAAA**AAAACTTCACAAAATGCTATACTAGGTAGG
Lactococcal P44	ACTC**TTGTTT**TACTTGATTTTATGTTAAAATAATTAATGAGTG
Lactococcal P59	ATTC**TTGACA**GGGAGAGATAGGTTTGATAGAATATAATAGTT
Leucocin A promoter	CATA**TTGATT**TAGAATACCTTTAGATATATAATTGAATGTGA
Carnobacteriocin A promoter	ATTT**TTGACT**AAATATCTATTTGGCATGATAGAATGTTCTTGTA
Brochocin-C promoter	TATT**TTGAGA**AATATTAACCAATAGTAAAAATTATCATGCTAT
Consensus sequence	NNNN**TTGACA**NNNNNNNNNNNNNNNNNTATAATNNNNNN

The brochocin-C construct was inserted downstream of promoters P21, P23, P32, and P44 in pGKV221, pGKV223, pGKV232, and pGKV244, respectively, (new plasmids named: pGB21, pGB23, pGB32, and pGB44) and the amount of brochocin-C activity produced by *C. maltaromaticum* LV17C transformed with pGB21, pGB23, pGB32, or pGB44 were determined using the spot-on-lawn assay. There were no detectable differences in the amount of brochocin-C produced by strains of *C. maltaromaticum* LV17C transformed with plasmids containing the promoters from *L. lactis* subsp. *cremoris* Wg2. There was 150 AU ml^{-1} of bacteriocin activity detected when transcription of brochocin-C was under the control of each of the 4 different promoters. Although these promoters showed different strengths based on

Table 4.4. Chloramphenicol resistance of *C. maltaromaticum* LV17C containing different promoters isolated from lactic acid bacteria and brochocin-C production determined using the spot-on-lawn assay.

Promoter	**Promoter description**	**Cm^R (μg ml^{-1})^a**	**Brochocin-C production (AU ml^{-1})^b**
P21	promoter from *L. lactis* subsp. *cremoris* Wg2	25	150
P23	promoter from *L. lactis* subsp. *cremoris* Wg2	30	150
P32	promoter from *L. lactis* subsp. *cremoris* Wg2	20	150
P44	promoter from *L. lactis* subsp. *cremoris* Wg2	20	150
P59	promoter from *L. lactis* subsp. *cremoris* Wg2	< 5	ND^c
PC1	promoter from pCaT, upstream of *cat* gene	< 5	150
PL1	promoter from *L. gelidum* UAL187, upstream of leucocin A genes	< 5	150
P1	native promoter fragment from *C. maltaromaticum* LV17C	10	150
P2	native promoter fragment from *C. maltaromaticum* LV17C	5	300
P3	native promoter fragment from *C. maltaromaticum* LV17C	20	300
P4	native promoter fragment from *C. maltaromaticum* LV17C	5	150
P5	native promoter fragment from *C. maltaromaticum* LV17C	10	150
P6	native promoter fragment from *C. maltaromaticum* LV17C	5	150
P8	native promoter fragment from *C. maltaromaticum* LV17C	20	150
P9	native promoter fragment from *C. maltaromaticum* LV17C	20	150
P10	native promoter fragment from *C. maltaromaticum* LV17C	20	150
P11	native promoter fragment from *C. maltaromaticum* LV17C	40	150
P12	native promoter fragment from *C. maltaromaticum* UAL26	10	150
P13	native promoter fragment from *C. maltaromaticum* UAL26	< 5	150

[a] Cm^R, chloramphenicol resistance, [b] AU ml^{-1}, arbitrary units per milliliter, [c] ND, not done

chloramphenicol resistance there was no detectable difference in brochocin-C production (Table 4.4). In other studies these promoters have been found to be effective for achieving differing amounts of proteins produced heterologously. Mesentericin Y105, an anti-*Listeria* bacteriocin, under the control of P59 was heterologously produced by *Leuconostoc mesenteroides* subsp. *dextanicum* and *Lc. cremoris* at a level similar to that of the natural producer (Biet et al., 2002). A 27-fold increase in chitinase activity was obtained using P59 compared to a 9-fold increase using P32 when the chitinase gene was expressed by *L. lactis* (Brurberg et al., 1994). Likewise, P59 was stronger than P23 in *L. lactis* for expressing the *emm6* gene for the M6 cell envelope-associated protein from *Streptococcus pyogenes* (Piard et al., 1997). However, both P21 and P59 yielded significantly lower endolysin activity compared to P32 when the genes for *Listeria monocytogenes* bacteriophage lysins were introduced into *L. lactis* (Gaeng et al., 2000). P59 also produced lower lipolytic activity compared to P44 and P23 when these promoters were used for the expression of the *lip* gene from *Staphylococcus hyicus* in *L. lactis* (Drouault *et al.*, 2000). Although it seems that the lactococcal promoters had different activity depending on the heterologous genes used when they were tested in *L. lactis* strains, in this study the different promoters from *L. lactis* did not appear to have any effect on brochocin-C production by *C. maltaromaticum* LV17C.

Since the lactococcal promoters were not effective for increasing brochocin-C production several other promoters were screened for activity in *C. maltaromaticum* LV17C and subsequently tested for their effect on bacteriocin production. Promoters from other LAB including bacteriocin producers as well as the promoter region from *B. campestris* ATCC 43754 were investigated to further explore the possibility of enhancing the heterologous production of brochocin-C. The nucleotide sequences of the promoter regions upstream of the various bacteriocin genes are shown in Table 4.3. The uncharacterized promoter region upstream of the *cat* gene in pCaT isolated from *Lb. plantarum* caTC2R was also tested for activity in *C. maltaromaticum* LV17C. A region upstream to the *cat* gene was amplified using PCR, digested, ligated into the promoter-screening vector, pGKV210, and transformed into *C. maltaromaticum* LV17C. Although the transformants did not exhibit resistance to chloramphenicol (Table 4.4) the presence of the *cat* promoter region was verified through PCR and the brochocin-C construct was successfully ligated downstream of this promoter (pGCB1). The amount of brochocin-C activity was not substantially different (150 AU ml^{-1}) from the amounts produced using the *L. lactis* subsp. *cremoris* Wg2 promoters (Table 4.4). The promoter region upstream of the leucocin A genes was amplified by PCR and these transformants also did not show resistance to chloramphenicol (Table 4.4) even though the –10 and –35 regions closely matched the consensus sequences (Table 4.3). The presence of the leucocin A promoter in pGKV210 was verified using PCR and the brochocin-C construct was ligated downstream of this promoter (pGLB1). The leucocin A promoter did not enhance the heterologous production of brochocin-C by *C. maltaromaticum* LV17C with 150 AU ml^{-1} of bacteriocin detected (Table 4.4).

The promoter region upstream of the carnobacteriocin A genes (Table 4.2) could not be amplified from plasmid isolations from *C. maltaromaticum* LV17A using the designed oligonucleotides. Further attempts to amplify this promoter region were not made since the lactococcal promoters, the promoters for leucocin A and the *cat* gene were not successful in terms of increasing detectable brochocin-C production. The promoter region upstream of the brochocin-C genes (Table 4.2) in *B. campestris* ATCC 43754 was amplified from chromosomal DNA, digested and ligated into pGKV210. After several attempts, chloramphenicol-resistant transformants could not be obtained. Because transformants were screened based on chloramphenicol resistance there is the possibility that the brochocin-C promoter was present in the vector but was not recognized by the *C. maltaromaticum* LV17C RNA polymerases. However, PCR amplification could not confirm the presence of the brochocin-C promoter due to non-specific amplification and further attempts to insert the promoter into the screening vector were not pursued.

Additional attempts to enhance the heterologous production of brochocin-C involved the isolation of native promoters from *C. maltaromaticum* LV17C and *C. maltaromaticum* UAL26 chromosomal DNA. Isolation of promoters from *C. maltaromaticum* LV17C and UAL26 were likely to have the most promise for increasing the expression of the brochocin-C genes since they would likely be recognized by the *C.*

maltaromaticum LV17C RNA polymerases. Digested chromosomal fragments were ligated into the promoter-screening vector and transformants were screened for resistance to chloramphenicol. Several transformants demonstrated various levels of chloramphenicol resistance (Table 4.4). Even though resistance ranged from less than 5 μg ml^{-1} up to 40 μg ml^{-1} chloramphenicol, brochocin-C activity was not substantially different (Table 4.4) for the majority of strains when the brochocin-C construct was inserted downstream of the various promoters in pGKV210, (pGPB1, pGPB4 to pGPB6, pGPB8 to pGPB13) and the strains were tested for bacteriocin production. However, two of the strains, *C. maltaromaticum* LV17C + pGPB2 and *C. maltaromaticum* + pGPB3 containing P2 and P3 promoters, respectively, did produce more bacteriocin with 300 AU ml^{-1} of bacteriocin detected (Table 4.4). One of these promoters was relatively weak with a chloramphenicol resistance level of only 5 μg ml^{-1} whereas the other promoter was quite strong with a resistance level of 20 μg ml^{-1} (Table 4.4). It was of particular interest that even though strains containing these promoters demonstrated very different amounts of chloramphenicol resistance they both produced the same amount of brochocin-C activity.

Perhaps using a reporter gene such as *cat-86*, which originated from *Bacillus pumilus* (van der Vossen et al., 1985) does not provide a good indication of promoter activity on bacteriocin genes. Recently, the *alr* gene, encoding alanine racemase, was used as a promoter-screening tool to study gene expression and control and for the identification of promoters in *Lb. plantarum* (Bron et al., 2004). Use of this type of promoter-screening tool could be investigated for the isolation of additional native promoters from *Carnobacterium* spp. Quantitative selection of promoters using the *alr* gene was achieved with increasing concentrations of D-cycloserine, an Alr inhibitor (Bron et al., 2004). This type of reporter gene may provide a better indication of promoter activity on bacteriocin genes compared with the *cat*-86 gene.

There is also the possibility that export of the bacteriocin via the Sec pathway is the limiting factor. The signal peptide of divergicin A is expressed along with the brochocin-C genes allowing the bacteriocin to be exported by the general secretion pathway rather than by a dedicated transport mechanism. Using the Sec pathway for the heterologous production of bacteriocins is advantageous because very little additional genetic material needs to be included along with the bacteriocin genes. Use of a dedicated pathway would necessitate the inclusion of much more genetic information in the bacteriocin construct to provide the genes encoding the ABC transporter and accessory proteins. Use of the Sec pathway for the heterologous production of other bacteriocins has previously proven to be successful (Biet et al., 1998; Biet et al., 2002; Franz et al., 1999; McCormick et al., 1996; McCormick et al., 1998; McCormick et al., 1999). However, when the Sec pathway is utilized for transport of bacteriocins out of the cell the amount of bacteriocin activity has been reduced compared with the production by the wild-type organisms (Biet et al., 1998; McCormick et al., 1996). It is possible that the Sec pathway reaches a certain capacity and the amount of bacteriocin exported via this route can not be increased.

It remains to be determined whether the promoters used in this study were modulating expression of the brochocin-C genes or whether gene expression remained constant regardless of promoter strength as determined by resistance to chloramphenicol.

4.4 References

Ahn, C., and Stiles, M.E. (1990a) Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. *J Appl Bacteriol* **69**: 302-310.

Ahn, C., and Stiles, M.E. (1990b) Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. *Appl Environ Microbiol* **56**: 2503-2510.

Ahn, C., Collins-Thompson, D., Duncan, C., and Stiles, M.E. (1992) Mobilization and location of the genetic determinant of chloramphenicol resistance from *Lactobacillus plantarum* caTC2R. *Plasmid* **27**: 169-176.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., (eds) (1995) *Short Protocols in Molecular Biology.* New York, New York: John Wiley and Sons Inc.

Biet, F., Berjeaud, J.M., Worobo, R.W., Cenatiempo, Y., and Fremaux, C. (1998) Heterologous expression of the bacteriocin mesentericin Y105 using the dedicated transport system and the general secretion pathway. *Microbiology* **144 (Pt 10)**: 2845-2854.

Biet, F., Cenatiempo, Y., and Fremaux, C. (2002) Identification of a replicon from pTXL1, a small cryptic plasmid from *Leuconostoc mesenteroides* subsp. *mesenteroides* Y110, and development of a food-grade vector. *Appl Environ Microbiol* **68**: 6451-6456.

Bron, P.A., Hoffer, S.M., Van Swam, I.I., De Vos, W.D., and Kleerebezem, M. (2004) Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. *Appl Environ Microbiol* **70**: 310-317.

Brurberg, M.B., Haandrikman, A.J., Leenhouts, K.J., Venema, G., and Nes, I.F. (1994) Expression of a chitinase gene from *Serratia marcescens* in *Lactococcus lactis* and *Lactobacillus plantarum*. *Appl Microbiol Biotechnol* **42**: 108-115.

Drouault, S., Corthier, G., Ehrlich, S.D., and Renault, P. (2000) Expression of the *Staphylococcus hyicus* lipase in *Lactococcus lactis*. *Appl Environ Microbiol* **66**: 588-598.

Franz, C.M., Schillinger, U., and Holzapfel, W.H. (1996) Production and characterization of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. *Int J Food Microbiol* **29**: 255-270.

Franz, C.M., Worobo, R.W., Quadri, L.E., Schillinger, U., Holzapfel, W.H., Vederas, J.C., and Stiles, M.E. (1999) Atypical genetic locus associated with constitutive production of enterocin B by *Enterococcus faecium* BFE 900. *Appl Environ Microbiol* **65**: 2170-2178.

Gaeng, S., Scherer, S., Neve, H., and Loessner, M.J. (2000) Gene cloning and expression and secretion of *Listeria monocytogenes* bacteriophage-lytic enzymes in *Lactococcus lactis*. *Appl Environ Microbiol* **66**: 2951-2958.

Hastings, J.W., and Stiles, M.E. (1991) Antibiosis of *Leuconostoc gelidum* isolated from meat. *J Appl Bacteriol* **70**: 127-134.

Kahala, M., and Palva, A. (1999) The expression signals of the *Lactobacillus brevis* slpA gene direct efficient heterologous protein production in lactic acid bacteria. *Appl Microbiol Biotechnol* **51**: 71-78.

Leenhouts, K., Bolhuis, A., Boot, J., Deutz, I., Toonen, M., Venema, G., Kok, J., and Ledeboer, A. (1998) Cloning, expression, and chromosomal stabilization of the *Propionibacterium shermanii* proline iminopeptidase gene (pip) for food-grade application in *Lactococcus lactis*. *Appl Environ Microbiol* **64**: 4736-4742.

Leisner, J.J., Greer, G.G., and Stiles, M.E. (1996) Control of beef spoilage by a sulfide-producing *Lactobacillus sake* strain with bacteriocinogenic *Leuconostoc gelidum* UAL187 during anaerobic storage at 2 degrees C. *Appl Environ Microbiol* **62**: 2610-2614.

Luchansky, J.B., Glass, K.A., Harsono, K.D., Degnan, A.J., Faith, N.G., Cauvin, B., Baccus-Taylor, G., Arihara, K., Bater, B., Maurer, A.J., and et al. (1992) Genomic analysis of *Pediococcus* starter cultures used to control *Listeria monocytogenes* in turkey summer sausage. *Appl Environ Microbiol* **58**: 3053-3059.

McCormick, J.K., Worobo, R.W., and Stiles, M.E. (1996) Expression of the antimicrobial peptide carnobacteriocin B2 by a signal peptide-dependent general secretory pathway. *Appl Environ Microbiol* **62**: 4095-4099.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E., and Van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. *Appl Environ Microbiol* **64**: 4757-4766.

McCormick, J.K., Klaenhammer, T.R., and Stiles, M.E. (1999) Colicin V can be produced by lactic acid bacteria. *Lett Appl Microbiol* **29**: 37-41.

Morgan, S.M., Galvin, M., Ross, R.P., and Hill, C. (2001) Evaluation of a spray-dried lacticin 3147 powder for the control of *Listeria monocytogenes* and *Bacillus cereus* in a range of food systems. *Lett Appl Microbiol* **33**: 387-391.

Muriana, P.M. (1996) Bacteriocins for control of *Listeria* spp. in food. *J Food Prot* **Supplement**: 54-63.

O'Keeffe, T., Hill, C., and Ross, R.P. (1999) Characterization and heterologous expression of the genes encoding enterocin a production, immunity, and regulation in *Enterococcus faecium* DPC1146. *Appl Environ Microbiol* **65**: 1506-1515.

Piard, J.C., Hautefort, I., Fischetti, V.A., Ehrlich, S.D., Fons, M., and Gruss, A. (1997) Cell wall anchoring of the *Streptococcus pyogenes* M6 protein in various lactic acid bacteria. *J Bacteriol* **179**: 3068-3072.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Scannell, A.G., Hill, C., Ross, R.P., Marx, S., Hartmeier, W., Elke, and Arendt, K. (2000a) Development of bioactive food packaging materials using immobilised bacteriocins lacticin 3147 and nisaplin. *Int J Food Microbiol* **60**: 241-249.

Scannell, A.G., Ross, R.P., Hill, C., and Arendt, E.K. (2000b) An effective lacticin biopreservative in fresh pork sausage. *J Food Prot* **63**: 370-375.

Schillinger, U., Kaya, M., and Lucke, F.K. (1991) Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. *J Appl Bacteriol* **70**: 473-478.

Siragusa, G.R., and Cutter, C.N. (1993) Brochocin-C, a new bacteriocin produced by *Brochothrix campestris*. *Appl Environ Microbiol* **59**: 2326-2328.

Solem, C., and Jensen, P.R. (2002) Modulation of gene expression made easy. *Appl Environ Microbiol* **68**: 2397-2403.

Talon, R., Grimont, P.A.D., Grimont, F., Gasser, F., and Boeufgras, J.M. (1988) *Brochothrix campestris* sp. nov. *Int J Syst Bacteriol* **38**: 99-102.

van Belkum, M.J., and Stiles, M.E. (1995) Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. *Appl Environ Microbiol* **61**: 3573-3579.

van der Vossen, J.M., Kok, J., and Venema, G. (1985) Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. *Appl Environ Microbiol* **50**: 540-542.

van der Vossen, J.M., van der Lelie, D., and Venema, G. (1987) Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. *Appl Environ Microbiol* **53**: 2452-2457.

Worobo, R.W., Henkel, T., Sailer, M., Roy, K.L., Vederas, J.C., and Stiles, M.E. (1994) Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. *Microbiology* **140 (Pt 3)**: 517-526.

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C., and Stiles, M.E. (1995) A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. *J Bacteriol* **177**: 3143-3149.

5. Real-time polymerase chain reaction to quantify brochocin-C expression in strains of *Carnobacterium maltaromaticum*

5.1 Introduction

A hurdle associated with the heterologous expression of bacteriocins by lactic acid bacteria is the reduction in bacteriocin activity compared with production by wild-type organisms. This effect was observed with the production of brochocin-C by *Carnobacterium* spp. (see Chapter 2). In an attempt to enhance brochocin-C activity, several promoters were analyzed. Although the promoters exhibited various strengths based on resistance to varying concentrations of chloramphenicol by strains of *C. maltaromaticum,* the amount of brochocin-C activity detected did not vary considerably (see Chapter 4). To determine if the promoters had an effect on brochocin-C gene expression it was necessary to quantify brochocin-C gene transcript levels.

A variety of methods such as Northern blotting, *in situ* hybridization, RNase protection assays, and PCR (Bustin, 2000; Giulietti et al., 2001; Schmittgen et al., 2000; Wang and Brown, 1999) have been used to quantify mRNA expression. However, these methods are laborious, require large amounts of total RNA, and post-PCR manipulation carries a risk for laboratory contamination (Giulietti et al., 2001). Real-time PCR is a relatively new technology that is preferred over other quantitative methods largely because results do not rely on end-point analyses that can be misleading due to product inhibition, enzyme instability and a decrease in the concentration of reaction components over time (Ramakers et al., 2003). Real-time PCR has many advantages over traditional methods for quantifying gene expression: multiple reactions can be run for any given biological treatment; the methodology is sensitive and therefore requires minimal sample; there is no need for postreaction manipulation which saves time and reduces the risk of laboratory contamination with PCR product; and real-time PCR is more precise, reproducible, and less labor-intensive (Edwards and Saunders, 2001; Winer et al., 1999). In addition, real-time monitoring of the PCR reaction allows faster and more accurate measurement of gene expression than quantifying mRNA using conventional reverse transcription PCR (Yin et al., 2001) and is quickly becoming the method of choice for the quantification of gene expression (Peirson et al., 2003) in both eukaryotic and prokaryotic systems.

With real-time PCR it is possible to measure the number of cycles necessary to detect a fluorescent signal and use this threshold cycle to determine the amount of a mRNA, which is reverse transcribed into cDNA, in a sample (Lekanne Deprez et al., 2002). Fluorescent signal from each PCR reaction is plotted versus the cycle number (Johnson et al., 2000) in 'real' time and reactions can be monitored instantaneously. Real-time PCR is based on the fact that the amount of amplified target is directly proportional to the input amount of target during the exponential range of amplification (Schmittgen et al., 2000). A fluorescence signal threshold is determined during this exponential phase of a PCR experiment at which point samples can be compared (Ginzinger, 2002) and this cycle threshold (C_T) is calculated as a function of the amount of background fluorescence. C_T is plotted at the point in which the signal generated from a sample is significantly greater than the background fluorescence (Ginzinger, 2002). Thus, the C_T has been defined as the PCR cycle in which the gain in fluorescence generated by the accumulating specific product exceeds 10 times the standard deviation of the mean baseline fluorescence between cycles 3 to 15 (Giulietti et al., 2001; Jung et al., 2000). The C_T value is inversely proportional to the input target mRNA level. A greater quantity of input mRNA target results in a lower C_T value because it requires fewer PCR cycles for fluorescent emission intensity to reach the threshold (Schmittgen et al., 2000; Winer et al., 1999).

For most research applications a relative approach to quantification is more practical than absolute quantification since relative quantification compares experimental samples against a control sample directly (Peirson et al., 2003). Original methods of relative quantification determination were based on the assumption of ideal amplification efficiencies with a doubling of product every cycle which allowed for the change in signal to be calculated using the formula $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (calibrator) and ΔC_T is the C_T of the target gene subtracted from the C_T of the reference gene (Giulietti et al., 2001; Johnson et al., 2000; Livak and Schmittgen, 2001; Peirson et al., 2003; Schmittgen et al., 2000; Winer et al., 1999). This $\Delta\Delta C_T$ method uses a single sample, termed the calibrator or control sample, for comparison with every unknown sample's gene expression level (Giulietti et al., 2001; Winer et al., 1999). Data analysis using this comparative $\Delta\Delta C_T$ method has an advantage over the relative and absolute standard curve methods since no standard curves that require the development of accurate RNA standards, which can be a time-consuming, labor-intensive, and expensive process, are required (Johnson et al., 2000; Schmittgen et al., 2000). A validation experiment must be performed to demonstrate that the amplification efficiencies of the target and reference genes are approximately equal (Johnson et al., 2000; Schmittgen et al., 2000) because the comparative $\Delta\Delta C_T$ method is very sensitive to variations in PCR efficiency (Ramakers et al., 2003). Quantification using the $\Delta\Delta C_T$ method has been found to be highly reproducible with low inter- and intra-assay variations. This accuracy has been attributed to the fact that C_T values are determined in the logarithmic phase of the PCR reaction where none of the reaction components are limiting (Johnson et al., 2000). The $\Delta\Delta C_T$ method has recently been refined to include the actual amplification efficiencies of the target and reference genes as calculated from cDNA standard curves (Pfaffl, 2001). Using this refined method, the relative expression ratio of a target gene is computed based on its real-time PCR efficiency and the crossing point (CP) or C_T difference of an unknown sample versus a control (Pfaffl, 2001; Pfaffl et al., 2002).

The reliability of relative comparisons using real-time PCR depends largely on the normalization of unwanted variations between samples; therefore, constantly expressed genes such as housekeeping genes are used as internal controls (reference genes) for normalization of the results (Giulietti et al., 2001; Pfaffl et al., 2002; Savli et al., 2003). Choosing the ideal housekeeping gene for each experiment is very important to ensure the credibility of the results (Giulietti et al., 2001) generated using comparative quantification. The ideal reference gene should consistently be expressed in all samples and should not be influenced by experimental conditions. In addition to normalization with a reference gene, PCR efficiency in real-time PCR has a major impact on the accuracy of the calculated expression results and the refined model (Pfaffl, 2001), with a correction for efficiency, results in a more reliable estimation of the 'real' expression ratio compared to a method with no efficiency correction (Pfaffl et al., 2002).

The objective of this study was to compare brochocin-C gene expression under the control of the various promoters using real-time PCR to determine if the level of bacteriocin transcript varied among strains and if gene expression correlated with detection of activity. Results of this study provide information on whether the use of various promoters is a viable option for increasing heterologous bacteriocin expression and/or whether other factors such as export via the Sec pathway may be a limitation.

5.2 Materials and methods

5.2.1 *Bacterial strains and growth conditions*

The bacterial strains used in this study are listed in Table 5.1. *Carnobacterium maltaromaticum* strains were grown in Brain Heart Infusion (BHI) broth (Difco Laboratories, Becton Dickinson Microbiology Systems, Sparks, Maryland) at 25°C. Erythromycin (Sigma-Alrich Canada Ltd., Oakville, Ontario) at a concentration of 5 μg ml^{-1} was added as a selective agent to ensure maintenance of the plasmids. Stock cultures were maintained at -70°C in broth media containing 20% (vol vol^{-1}) glycerol.

Frozen stock cultures of strains of *C. maltaromaticum* with the brochocin-C construct under the control of different promoters (see Chapter 4) were inoculated into 10 ml of BHI broth containing erythromycin (BHI + Em^5) and incubated at 25°C for 24 h. One hundred microliters of each fully-grown culture (ca. log 9 cfu ml^{-1}) was transferred into fresh BHI + Em^5 and incubated at 25°C for 24 h. This procedure was repeated once more prior to preparing the cultures for the start of the experiment. To rejuvenate and synchronize cultures to an active mode of growth, 1 ml of fully-grown culture was centrifuged at 10,000 x *g* for 1 min and the supernatant was discarded. One milliliter of BHI + Em^5 was removed from tubes containing 10 ml of broth and was used to resuspend the cell pellet. The resuspended pellet was added back to the remaining 9 ml of BHI +

Table 5.1. Bacterial strains used in this study and a description of their relevant characteristics including level of resistance to chloramphenicol when various promoters were used.

Bacterial Strain	**Relevant Characteristics**[a]
Carnobacterium maltaromaticum LV17C +pGKV210	bacteriocin negative, promoterless, Em^{R}
C. maltaromaticum LV17C + pGPB1	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (10 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB2	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (5 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB3	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (20 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB4	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (5 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB5	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (10 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB6	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (5 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB8	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (20 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB9	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (20 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB10	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (20 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB11	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* LV17C, Cm^{R} (40 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB12	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* UAL26, Cm^{R} (10 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGPB13	produces brochocin-C, contains uncharacterized native promoter from *C. maltaromaticum* UAL26, Cm^{R} (<5 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGCB1	produces brochocin-C, contains promoter upstream of the *cat* gene from pCaT plasmid in *Lactobacillus plantarum* caTC2R, Cm^{R} (<5 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGLB1	produces brochocin-C, contains promoter upstream of leucocin A genes from *Leuconostoc gelidum* UAL187, Cm^{R} (<5 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGB21	produces brochocin-C, contains P21 promoter from pGKV221, Cm^{R} (25 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGB23	produces brochocin-C, contains P23 promoter from pGKV223, Cm^{R} (30 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGB32	produces brochocin-C, contains P44 promoter from pGKV232, Cm^{R} (20 μg/ml), Em^{R}
C. maltaromaticum LV17C + pGB44	produces brochocin-C, contains P44 promoter from pGKV244, Cm^{R} (20 μg/ml), Em^{R}

[a] Em^{R}, erythromycin resistant; Cm^{R}, chloramphenicol resistant

Em^5 and incubated at 25°C for 3 h. This procedure was repeated once more and 10 µl of the culture was inoculated into each of 3 tubes of 10 ml BHI + Em^5 (2 tubes for freezing and 1 tube to determine optical density). The cultures were incubated at 25°C and the optical density at 600 nm (OD_{600}) was determined to obtain cultures that were in the late exponential growth phase. Cultures at an OD_{600} of 0.30 to 0.40 were immediately placed on ice and centrifuged at 2,500 x *g* for 10 min at 4°C. The supernatant fluids were discarded and the cell pellets were rapidly frozen in supercooled 95% ethanol (-80°C) and the frozen pellets were stored at -80°C until RNA isolations were conducted.

5.2.2 *Primer design*

Oligonucleotides for amplification of brochocin-C (target gene) and 16S rRNA (reference gene) were designed for use on the LightCycler II (Roche Molecular Biochemicals, Mannheim, Germany) using LightCycler Probe Design Software (version 3.5, Roche). The sequences of the primers used for the amplification of the brochocin-C gene (McCormick et al., 1998) were 5'-AGCTTCTGCTTACAGTTCA (BrcC1) that included the nucleotides for the end of divergicin A signal peptide and the start of the b*rcA* gene and 5'-GCTCCTAGTTACCTAATAATCCAC (BrcC2) that included the nucleotides at the end of *brcA*. An amplicon of 194 bp was produced. The oligonucleotides for amplification of 16S rRNA were 5'-ACAAAGTGACAGGTGG (RNA5) at nucleotide 1029, 5'-AGATTAGCTTGACCTCGC (RNA6) at nucleotide 1274 and were based on the 1514 bp sequence of *Carnobacterium* sp. 16S rRNA [National Center for Biotechnology Information (NCBI) GenBank, Accession No. CSP427446]. These primers produced an amplicon of 247 bp.

5.2.3 *RNA Isolation*

Total RNA was isolated from the strains of carnobacteria using the method described by Cheung et al. (1994) with the following modifications. Frozen bacterial cell pellets were placed on ice and immediately resuspended in 1 ml of extraction reagent. The extraction reagent consisted of 40% water saturated RNA phenol (Stratagene, La

Jolla, California), 0.6% cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich Canada Ltd., Oakville, Ontario), 50 mM sodium acetate (pH 4.5), and 1 mM dithiothreitol. Care was taken to ensure that the cell pellets did not thaw prior to the addition of the extraction reagent. The thawed and mixed cell suspensions were transferred to 2 ml screw cap microfuge tubes containing 0.5 ml dimethyldichlorosilane (DMCS) treated glass beads (100/120 mesh, Alltech Associates Inc. Deerfield, Illinois). The tubes were shaken for 20 sec on setting 6.0 on a FastPrep instrument (model FP120; Savant Instruments Inc., Holbrook, New York) and immediately placed on ice. Tubes underwent a pulse spin to facilitate the settling of the beads. The supernatants were transferred to new tubes containing 200 µl chloroform, vortexed for 15 sec and incubated at room temperature for 3 min. The mixtures were centrifuged at 12,000 x *g* for 15 min at 4°C and the aqueous layers were withdrawn to new tubes containing 200 µl chloroform and a second extraction step was conducted. The RNA in the aqueous layers were precipitated with 500 µl isopropanol for 10 min on ice and pelleted at 12,000 x *g* for 10 min at 4°C. The pellets were washed with 700 µl of 75% ethanol and dried under vacuum at 45°C (Centrivap Console, Labconco Corporation, Kansas City, Missouri) for no more than 3 min to ensure that the RNA pellets were not overdried.

The RNA pellets were each resuspended in 40 µl *DNase* I digestion reagent which consisted of DEPC treated water, 1X *DNase* I reaction buffer, and 4 U *DNase* I, (amplification grade; Invitrogen Canada Inc., Burlington, Ontario). The resuspended pellets were incubated for 30 min at room temperature and the *DNase* I was inactivated by the addition of 2.5 mM EDTA followed by heating for 10 min at 65°C. The RNA was precipitated overnight at -20°C using 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol. The precipitated RNA was centrifuged at 12,000 x *g* for 30 min at 4°C. The pellets were washed with 700 µl of 75% ethanol and dried under vacuum at 45°C for no more than 3 min to ensure that the pellets were not overdried. The dried RNA pellets were resuspended in 40 µl DEPC-treated sterile purified water and stored at -80°C.

Prior to storage at -80°C, the RNA from carnobacteria was analyzed using conventional PCR to verify the absence of contaminating DNA. Five microliters of each RNA extraction was added to each of 45 µl of PCR mixture containing 1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates (Invitrogen Canada Inc.), 1 U Platinum® *Taq* DNA polymerase (Invitrogen Canada Inc.) and 1 µM each of brochocin-C or 16S rRNA primers. Amplification was conducted on a DNA Engine® Peltier Thermal Cycler (PTC-200, MJ Research Inc., Waltham, Massachusetts) with 32 cycles (denaturation, 94°C, 1 min, annealing, 55°C, 1 min, extension, 72°C, 1 min) followed by a final extension step at 72°C for 10 min. The PCR products were visualized on ethidium bromide stained 1.2 % agarose gels.

5.2.4 *Reverse transcription*

RNA concentration was determined spectrophotometrically (GeneQuant II, Pharmacia Biotech, Cambridge, England) by measuring the A_{260}/A_{280} ratio of a 1:10 dilution of RNA in DEPC-treated water.

First strand cDNA synthesis was conducted according to the manufacturer's instructions using the 1st Strand cDNA Synthesis Kit for RT-PCR that contained Avian Myeloblastosis Virus (AMV) reverse transcriptase (Roche Diagnostics Corp., Indianapolis, Indiana). Random primer $p(dN)_6$ was used in the reverse transcription reactions. The appropriate amount of RNA was added to freshly prepared reverse transcription reaction mixtures to obtain approximately 1 µg of total RNA per 20 µl reaction volume.

The cDNA from all the strains was analyzed using conventional PCR to verify the presence of the brochocin-C and 16S rRNA genes. Five microliters of each cDNA extraction was added to each of 45 µl of PCR mixture containing 1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates (Invitrogen Canada Inc.), 1 U Platinum® *Taq* DNA polymerase (Invitrogen Canada Inc.) and 1 µM each of brochocin-C or 16S rRNA primers. Amplification was conducted on a DNA Engine® Peltier Thermal Cycler with 32 cycles (denaturation, 94°C, 1 min, annealing, 55°C, 1 min, extension, 72°C, 1 min) followed by a final extension step at 72°C for 10 min. The PCR products were visualized on ethidium bromide stained 1.2 % agarose gels.

5.2.5 *Real-time PCR*

Real-time PCR reactions were performed in the LightCycler using the LightCycler-FastStart DNA Master SYBR Green I PCR kit (Roche) as recommended by the manufacturer. The DNA binding dye, SYBR Green I, binds in the minor groove of double-stranded DNA in a sequence-independent manner and when it binds to DNA its fluorescence increases over 100-fold compared to when it is in solution (Lekanne Deprez et al., 2002). SYBR Green I is excited at a wavelength of 497 nm and detected at a wavelength of 521 nm. SYBR Green I is the least expensive option for performing kinetic PCR assays since there is no requirement for amplicon-specific probes (Bustin, 2000); however, both desired and undesired products will generate a signal.

The LightCycler is an air-heated system that performs PCR reactions in glass capillaries that hold up to 20 µl of sample (32 samples/run) which allows for very rapid cycling due to the favorable surface-to-volume ratio of the capillaries (Giulietti et al., 2001; Jung et al., 2000). Fluorescence readings are taken during every cycle of the PCR reaction and are immediately displayed after each measurement thereby allowing for amplification runs to be terminated or extended during individual runs (Bustin, 2000).

To optimize the PCR reactions, three concentrations of $MgCl_2$ (3, 4, and 5 mM) were tested with primers for the brochocin-C and 16S rRNA genes using 100 ng ml^{-1} *C. maltaromaticum* LV17C + pGB32 cDNA as the template. The melting curves were analyzed to ensure the presence of only the desired products and the absence of primer dimers.

The efficiency of the PCR for the brochocin-C and 16S rRNA genes were obtained by spectrophotometrically determining the cDNA concentrations of two arbitrarily chosen strains (*C. maltaromaticum* LV17C + pGPB11 and *C. maltaromaticum* LV17C + pGB32) and preparing serial 10-fold dilutions using sterile purified water. Real-time PCR on the LightCycler was conducted using 400, 40, 4, 0,4, 0.04, and 0.004 ng cDNA, 3 mM $MgCl_2$, 0.5 µM BrcC or RNA primers, and 2 µl SYBR Green I per 20 μl total volume. The crossing points (CPs) were plotted against the concentrations of cDNA and the slope and R^2 values were calculated using linear regression analysis (Microsoft® Excel 2000). The PCR efficiencies were determined using the formula: $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001) and the average PCR efficiency for each gene was used in all subsequent relative quantification calculations.

The concentrations of cDNA from the remaining strains were spectrophotometrically determined and the appropriate dilutions were made to obtain a final concentration of approximately 100 ng ml^{-1} cDNA. Real-time PCR was conducted on cDNA from all the strains using 0.4 ng cDNA, 3 mM $MgCl_2$, 0.5 μM BrcC or RNA primers, and 2 μl SYBR Green I per 20 μl total volume. The cDNA was amplified using the following temperature profiles: denaturation for 1 cycle at 95°C for 3 min (temperature transition, 20°C sec^{-1}) followed by 50 cycles at 95°C for 5 sec (temperature transition, 20°C sec^{-1}), 57°C for 8 sec (temperature transition, 20°C sec^{-1}), and 72°C for 25 sec (temperature transition, 3°C sec^{-1}). Melting curve analyses were done using 1 cycle of 95°C for 0 sec (temperature transition, 20°C sec^{-1}), 67°C for 30 sec (temperature transition, 20°C sec^{-1}), and 95°C for 0 sec (temperature transition, 0.1°C sec^{-1}) followed by 1 cooling cycle of 40°C for 30 sec (temperature transition, 20°C sec^{-1}).

5.2.6 *Relative quantification*

Relative quantification of brochocin-C expression was calculated using the formula proposed by Pfaffl (2001) which provides the relative expression ratio (R) of a target gene (brochocin-C) compared to a reference or housekeeping gene (16S rRNA) and is based on the PCR efficiencies (E) for each gene and the crossing point (CP) deviation (ΔCP) of the sample strain versus a control (calibrator) strain. The model used was:

$$R = \frac{E_{(\text{target})}^{\Delta CP_{\text{target}}(\text{control} - \text{sample})}}{E_{(\text{ref})}^{\Delta CP_{\text{ref}}(\text{control} - \text{sample})}}$$

The ΔCP_{target} was the CP for the brochocin-C genes from each of the strains subtracted from the CP of the brochocin-C gene from *C. maltaromaticum* LV17C + pGPB6, the strain arbitrarily chosen to serve as the control. The ΔCP_{ref} was the CP for the 16S rRNA genes from each of the strains subtracted from the CP for the 16S rRNA gene from *C. maltaromaticum* LV17C + pGPB6.

The relative expression ratios determined using the Pfaffl method were compared to the ratios determined using the conventional comparative $\Delta\Delta C_T$ method to determine if there was a substantial difference between the two methods of calculation. The amount of target, which is normalized to the housekeeping gene and relative to the calibrator, is given by the formula $R = 2^{\Delta\Delta C_T}$. $\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (calibrator) and ΔC_T is the C_T of the target gene subtracted from the C_T of the reference gene (Giulietti et al., 2001). This method can be can be used if the PCR efficiencies of the target gene and reference gene are approximately equal. It was necessary to perform three separate real-time PCR runs to include all of the strains. Each of the runs included cDNA from *C. maltaromaticum* LV17C + pGPB6 as the control.

5.3 Results and discussion

5.3.1 *RNA Isolation and reverse transcription*

The abundance of mRNA decreases as cells move from the exponential to the stationary growth phase (Shepard and Gilmore, 1999) and a rapid, consistent procedure for harvesting cells was essential to minimize the effects of mRNA instability. The mRNA in prokaryotes can have a very short half-life and it has been noted that the half-life of mRNA is much longer in fast-growing cells than in slow-growing cells (Carrier and Keasling, 1997; Grunberg-Manago, 1999). Vandecasteele et al. (2001) found that gene expression was 110- to 300-fold higher during exponential growth than gene expression during stationary phase. The majority of the strains reached the desired optical density after 12.5 to 13.5 hours of incubation at 25°C. Two of the strains, *C. maltaromaticum* LV17C + pGPB2 and *C. maltaromaticum* LV17C + pGPB3, took 15 hours to reach an OD_{600} of 0.30 and 0.35, respectively. It was of interest that these two strains were subseqently determined by real-time PCR to have higher transcript levels of the brochocin-C gene compared to the other strains. These two strains also produced higher levels of brochocin-C activity (see Chapter 4) compared to the rest of the strains. The higher level of production of bacteriocin by these two strains appeared to have an effect on the cells resulting in a slower growth rate.

RNA isolated from all 19 strains was checked for the presence of contaminating DNA after treatment with *DNase* I by conventional PCR using brochocin-C and 16S rRNA primers. No amplification products were detected on 1.2% agarose gel indicating that contamination with DNA did not occur. The traditional method for assessing RNA purity and concentration is UV spectroscopy that measures absorbance of diluted RNA at 260 and 280 nm. The A_{260}/A_{280} ratios ranged from 1.71 to 1.79 and the concentrations of RNA ranged from approximately 400 to 1200 μg ml^{-1}. Total RNA isolated from *C. maltaromaticum* strains showed good purity based on A_{260}/A_{280} ratios. Typically, highly purified RNA should have an A_{260}/A_{280} ratio of 1.8 to 2.0. DEPC water was used as the diluent since it was also used to resuspend the RNA pellets. The A_{260}/A_{280} ratio is dependent on both the pH and ionic strength of the diluent with A_{280} decreasing as pH increases (Wilfinger et al., 1997); therefore use of DEPC, which is slightly acidic, resulted in lower A_{260}/A_{280} ratios. It has been shown that adjusting the pH of water for spectrophotometric analysis from approximately 5.4 to a pH of 7.5 to 8.5 changes the A_{260}/A_{280} ratios from 1.5 to 2.0 (Wilfinger et al., 1997). Therefore, although the ratios obtained in this study were lower than 1.8 to 2.0, the RNA would still be considered to be of high purity. Generally, LightCycler PCR is susceptible to traces of inhibitors; therefore, it is essential that RNA is purified carefully and thoroughly (Goerke et al., 2001). The RNA isolation method used in this study was relatively simple, fast, and resulted in good yields of purified total RNA due to the direct fixation of RNases, the absence of enzymatic digestion steps, and no interference of PCR reactions by inhibitors.

Appropriate amounts of RNA ranging from 1.0 to 2.5 μl were added to the reverse transcription (RT) mixtures to obtain approximately 1 μg of total RNA per 20 μl reaction volume. After reverse transcription the cDNA was checked by conventional

PCR using primers for the brochocin-C and 16S RNA genes. Amplification products of the correct size were observed for all 19 strains (data not shown). The RT step is critical for sensitive and accurate quantification and the amount of cDNA produced must accurately represent the RNA input amounts (Bustin, 2002). It was determined in this study that RT master mix can not be previously frozen prior to the addition of RNA. Storage at -20°C resulted in lower transciption efficiency based on higher crossing points obtained during real-time PCR (data not shown). The RT master mix must be prepared from newly thawed reagents, stored on ice, RNA added, and the appropriate incubation conditions carried out to obtain consistent results between real-time PCR runs.

5.3.2 *Real-time PCR*

To quantify the expression of brochocin-C under the control of the various promoters using real-time PCR, it was necessary to find a suitable reference gene. Normalization to a housekeeping gene is currently the most acceptable method to correct for minor variations in the reverse transcription reaction due to differences in input RNA amount and efficiency of reverse transcription (Giulietti et al., 2001). The proportion of constantly expressed genes (housekeeping genes) in the total cellular RNA is assumed to be equal between different samples and therefore normalization of sample values by means of a reference gene serves to eliminate sample-to-sample variation (Savli et al., 2003). There was no published information on housekeeping genes in *Carnobacterium* spp.; however, several housekeeping genes for other gram positive bacteria have been documented (Homan et al., 2002; Mora et al., 2000; Nallapareddy et al., 2002). Published oligonucleotide sequences for three housekeeping genes, glucose-6-phosphate dehydrogenase (*gdh*), ATP synthase (*atpA*), and adenylate kinase (*adk*), from *Enterococcus faecium* (Homan et al., 2002) were obtained to determine if amplication of these genes in two test strains, *C. maltaromaticum* LV17C and *C. maltaromaticum* LV17C + pGPB11, was possible. Multiple amplification products were obtained for *adk* and *atpA* using conventional PCR and only primer dimer was detected for *gdh* using real-time PCR even though this gene appeared promising using conventional PCR (data not shown). Many studies utilizing real-time PCR for eukaryotic systems rely on β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, and ribosomal RNA (rRNA) as reference genes (Giulietti et al., 2001; Liu and Saint, 2002; Livak and Schmittgen, 2001; Peirson et al., 2003; Sturzenbaum and Kille, 2001). The 28S and 18S rRNA have been recommended as internal controls for mRNA quantification studies because they compare very favorably in terms of steady-state expression levels when compared to other housekeeping genes (Sturzenbaum and Kille, 2001). In prokaryotic studies, 16S rRNA was used as a reliable reference gene (Edwards and Saunders, 2001; Rosato et al., 2003; Vandecasteele et al., 2001). Although the genomic sequence for *C. maltaromaticum* LV17C has not been published, sequences for 16S rRNA genes from other carnobacteria were available and were highly conserved among many strains of lactic acid bacteria when a basic local alignment search tool (BLAST; NCBI) analysis was conducted. Three sets of oligonucleotides designed to amplify portions of the 16S rRNA gene were designed and one of the sets (RNA5 and RNA6) produced one amplification product and no primer dimers using real-time PCR melting curve analysis (data not shown) and these primers were used in subsequent experiments. An important aspect for gene quantification using real-time PCR is to ensure that the gene chosen for normalization of RNA load, in this case 16S rRNA, is truly invariant throughout the experimental assay (Winer et al., 1999). The mean and standard error (SE) of the CPs (Table 5.2) for the 16S rRNA gene from cDNA from each bacterial strain was calculated for each of the 3 real-time PCR runs (6 cDNA for Run 1, 9 cDNA for Run 2, and 6 cDNA for Run 3) as well as the 3 runs combined (21 cDNA). Run 1 had a mean of 19.70 and SE of 0.11, run 2 had a mean of 19.87 with an SE of 0.45, and run 3 had a mean of 19.24 with an SE of 0.29. The mean CP for the 16S rRNA gene for all 3 runs combined was 19.64 and the SE was 0.21. The 16S rRNA gene was a good choice for use as a reference gene in *C. maltaromaticum* LV17C because there was relatively little variation in the amounts of transcript within PCR runs and between 3 separate runs.

Because real-time PCR is a highly sensitive technique it was necessary to optimize the PCR conditions prior to quantifying gene expression. There are many variables that can influence the efficiency of PCR amplifications (Wang et al., 1989). Some of the parameters that can be easily controlled include the concentrations of

Table 5.2. Relative quantification of brochocin-C gene expression and brochocin-C production by promoter-containing strains of *Carnobacterium maltaromaticum* LV17C.

Bacterial Strain	**Crossing Points**		**Relative Quantification Ratios**		**Brochocin-C Production (AU ml^{-1})^{a}**
	Brochocin-C	**16S rRNA**	**Pfaffle Method**	**$\Delta\Delta C_T$ Method**	
C. maltaromaticum LV17C + pGPB1	32.52	19.74	0.7	0.7	150
C. maltaromaticum LV17C + pGPB2	31.02	19.78	2.1	2.0	300
C. maltaromaticum LV17C + pGPB3	30.95	19.64	3.8	3.6	300
C. maltaromaticum LV17C + pGPB4	31.86	19.82	1.2	1.2	150
C. maltaromaticum LV17C + pGPB5	32.20	19.70	1.6	1.6	150
C. maltaromaticum LV17C + pGPB8	32.92	19.69	1.0	1.0	150
C. maltaromaticum LV17C + pGPB9	31.49	19.19	1.0	1.0	150
C. maltaromaticum LV17C + pGPB10	33.28	19.78	0.4	0.4	150
C. maltaromaticum LV17C + pGPB11	33.75	18.31	0.2	0.3	150
C. maltaromaticum LV17C + pGPB12	31.74	19.11	1.1	1.0	150
C. maltaromaticum LV17C + pGPB13	33.85	20.84	0.7	0.8	150
C. maltaromaticum LV17C + pGCB1	31.81	18.76	0.8	0.8	150
C. maltaromaticum LV17C + pGLB1	32.61	18.97	0.5	0.5	150
C. maltaromaticum LV17C + pGB21	32.20	18.49	0.5	0.5	150
C. maltaromaticum LV17C + pGB23	35.32	22.88	0.9	1.2	150
C. maltaromaticum LV17C + pGB32	31.45	18.32	1.1	1.0	150
C. maltaromaticum LV17C + pGB44	31.70	19.70	1.6	1.6	150

[a] AU ml^{-1}, arbitrary units per milliliter

template, dNTPs, $MgCl_2$, primers, polymerase, and the PCR cycle profile (Wang et al., 1989). Real-time PCR reactions were optimized using *C. maltaromaticum* LV17C + pGB32 cDNA to determine the best concentration of $MgCl_2$ to use for all subsequent PCR reactions. Amplification of brochocin-C and 16S rRNA genes for PCR reactions using 3, 4, and 5 mM $MgCl_2$ is shown in Figure 5.1.A. Figures 5.1.B and 5.1.C show the melting curve data for the PCR reactions including sterile purified water as negative controls. The amplification plot (Figure 5.1.A) showed that the highest fluorescence is reached when 3 mM of $MgCl_2$ was used and the melting curves (Figures 5.1.B and 5.1.C) demonstrated that the highest peak height was also achieved with 3 mM $MgCl_2$. The melting curve analysis also indicated that there was only one amplification product produced with no significant primer dimer formation when cDNA was included in the reactions. Primer dimer was only produced when purified water was used in the reactions. Melting curve analysis is essential when SYBR Green I is used because this dye detects all double-stranded DNA, including primer dimers and other undesired products. The melting curve of a product is dependent on GC content, length, and sequence, and therefore PCR products can be distinguished by their melting curves (Ririe et al., 1997). A product melting curve is obtained during real-time PCR by monitoring the fluorescence as the temperature passes through the product denaturation temperature and the melting curve can be used to differentiate amplification products separated by less than 2°C in melting temperature (Ririe et al., 1997). Because of length differences, nonspecific products usually melt at a lower temperature than the desired PCR products (Rasmussen et al., 1998; Ririe et al., 1997). In this study melting curve analysis showed a melting temperature of about 88°C for the brochocin-C gene and about 87.5°C for the 16S rRNA gene, whereas the primer dimers had much lower melting temperatures of about 75°C and 77°C, respectively (Figure 5.1.B and 5.1.C). Analysis of the melting peaks provides a greater level of certainty that the fluorescence derives from the intended product rather than from nonspecific amplification and also allows for product identification during PCR without subsequent electrophoresis (Ririe et al., 1997). In addition, careful design of primers and optimization of the reaction conditions can reduce the formation of primer dimers (Giulietti et al., 2001).

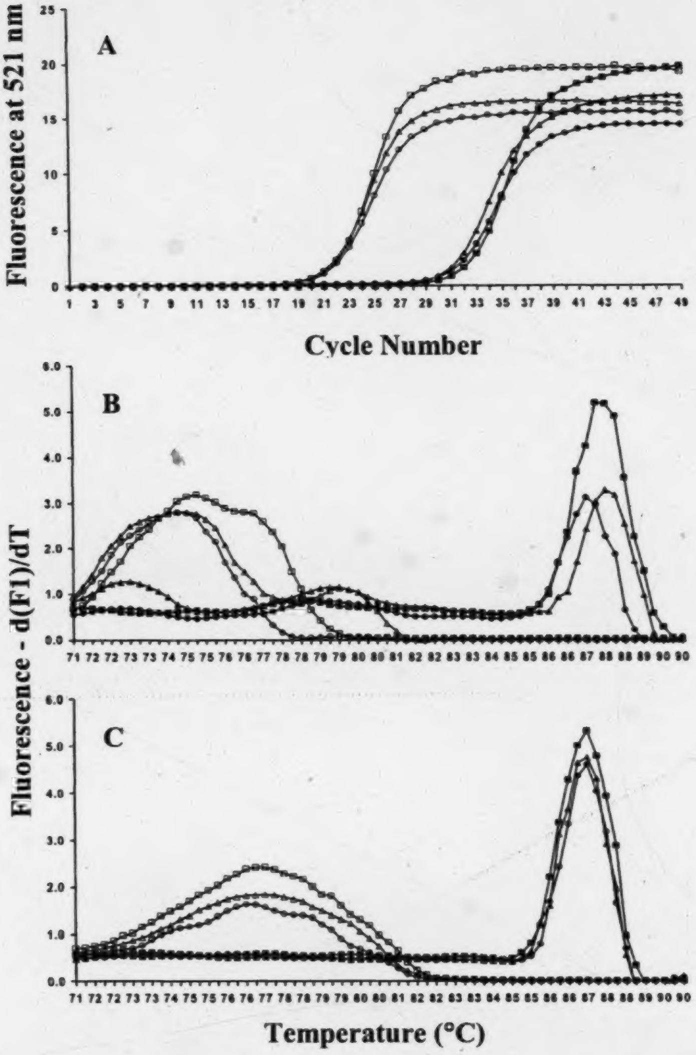

Figure 5.1. Amplification and melting curve analysis of brochocin-C and 16S rRNA gene expression using real-time PCR with three concentrations of $MgCl_2$.

■, 3 mM $MgCl_2$; ▲, 4 mM $MgCl_2$; ●, 5 mM $MgCl_2$. **A**, quantification of brochocin-C and 16S rRNA genes using 0.4 ng total cDNA from *C. maltaromaticum* LV17C + pGB32, closed symbols for brochocin-C gene, open symbols for 16S rRNA gene; **B**, melting curve analysis of brochocin-C gene using 0.4 ng total cDNA from *C. maltaromaticum* LV17C + pGB32, closed symbols for cDNA used as template, open symbols for water used as template; **C**, melting curve analysis of 16S rRNA using 0.4 ng total cDNA from *C. maltaromaticum* LV17C + pGB32, closed symbols for cDNA used as template, open symbols for water used as template.

PCR efficiencies were determined for amplification of the brochocin-C and 16S rRNA genes using various concentrations of cDNA from two strains, *C. maltaromaticum* LV17C + pGPB11 and *C. maltaromaticum* LV17C + pGB32. Amplification plots (Figure 5.2) were used to plot the crossing points versus the concentrations of cDNA (Figure 5.3) and the R^2 values and slopes were determined using linear regression analysis. The lowest concentration of cDNA (0.004 ng ml^{-1}) was not used in the determination of efficiency because melting curve analysis indicated that there was some primer dimer produced at this concentration for the brochocin-C gene (data not shown). The PCR efficiencies were determined using the formula: $E = 10^{(-1/slope)}$ (Pfaffl, 2001). The calculated PCR efficiencies for the brochocin-C gene were 2.09 for *C. maltaromaticum* LV17C + pGPB11 and 2.04 for *C. maltaromaticum* LV17C + pGB32. The PCR efficiency for the 16S rRNA gene was 1.92 for *C. maltaromaticum* LV17C + pGPB11 and *C. maltaromaticum* LV17C + pGB32. The PCR efficiency values used to calculate all subsequent relative expression ratios using the Pfaffl model were the average PCR efficiency (2.07) for the brochocin-C gene and 1.92 for the 16S rRNA gene. To verify that amplification efficiencies were not substantially different, the ΔC_T was plotted against the concentration of total cDNA. The ΔCP is the CP of the reference gene subtracted from the CP of the target gene for each concentration of total cDNA. The equation to the line is calculated by linear regression analysis and if the slope is zero, the amplification efficiencies of the target and reference genes are approximately equal (Johnson et al., 2000; Schmittgen et al., 2000). Linear regression analysis resulted in the equations: $y = 0.0349x + 13.794$ using cDNA from *C. maltaromaticum* LV17C + pGPB11 and $y = 0.095x + 10.655$ using cDNA from *C. maltaromaticum* LV17C + pGB32 (data not shown). Because the values are below the recommended value of <0.1 (Johnson et al., 2000), the amplification efficiencies for the brochocin-C and 16S rRNA genes would be considered to be similar.

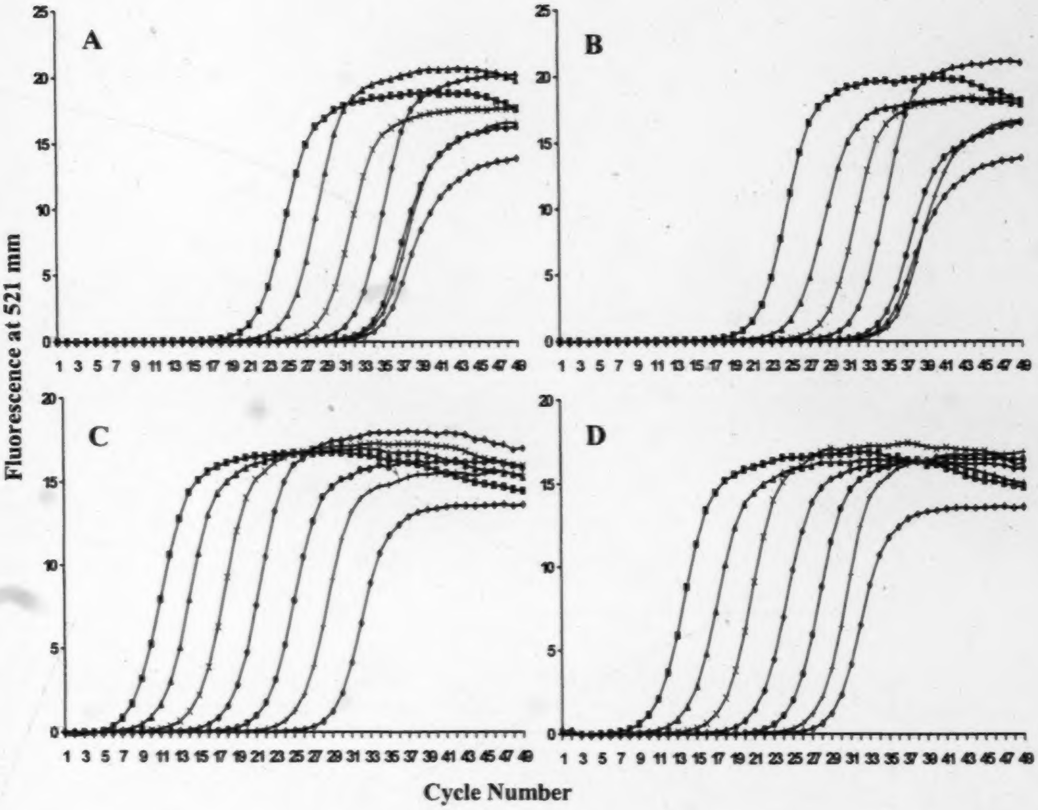

Figure 5.2. Amplification plots of brochocin-C and 16S rRNA genes. Serial dilutions of cDNA were amplified by real-time PCR using SYBR Green I.

A, quantification of brochocin-C gene using cDNA from *C. maltaromaticum* LV17C + pGPB11; **B**, quantification of brochocin-C gene using cDNA from *C. maltaromaticum* LV17C + pGB32; **C**. quantification of 16S rRNA gene using cDNA from *C. maltaromaticum* LV17C + pGPB11; **D**, quantification of 16S rRNA gene using cDNA from *C. maltaromaticum* LV17C + pGB32. ◇, water; ■, 400 ng ml^{-1} cDNA; ▲, 40 ng ml^{-1}; ×,4 ng ml^{-1};◆, 0.4 ng ml^{-1}; ●, 0.04 ng ml^{-1}; +, 0.004 ng ml^{-1}.

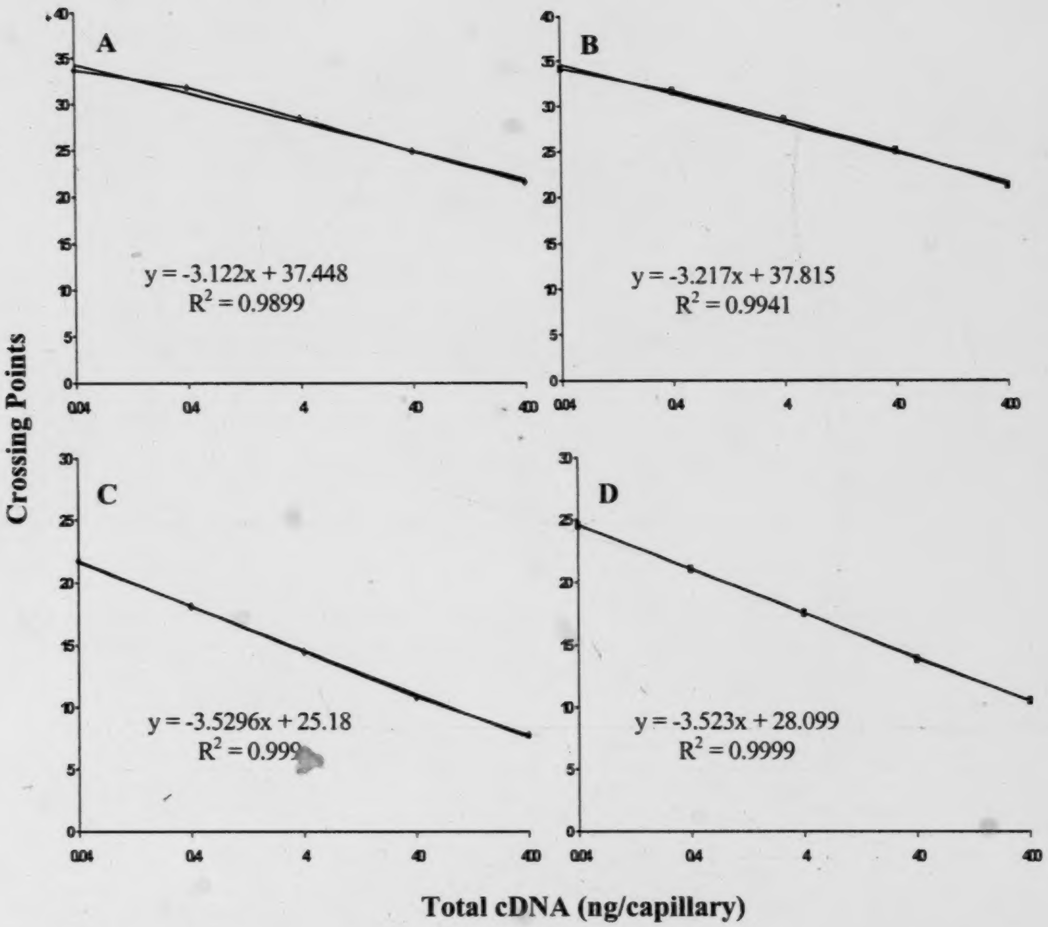

Figure 5.3. Standard curves for brochocin-C and 16S rRNA to determine PCR efficiency values. Crossing point values plotted against input cDNA concentrations. Linear regression analysis was used to determine slope and R^2 values. **A**, brochocin-C gene amplified from *C. maltaromaticum* LV17C + pGPB11; **B**, brochocin-C gene amplified from *C. maltaromaticum* LV17C + pGB32; **C**, 16S rRNA gene amplified from *C. maltaromaticum* LV17C + pGPB11; **D**, 16S rRNA gene amplified from *C. maltaromaticum* LV17C + pGB32.

Transcript levels of the brochocin-C gene under the control of various promoters in *C. maltaromaticum* LV17C were compared using one arbitrarily chosen strain, *C. maltaromaticum* LV17C + pGPB6, as the control (calibrator) strain. The transcript levels were normalized between strains using the 16S rRNA gene as the reference or housekeeping gene. Two models were used to calculate the relative expression ratios using the crossing points derived from the amplification of the brochocin-C and 16S rRNA genes. An example of the amplification for the brochocin-C and 16S rRNA genes from 3 of the strains is shown in Figure 5.4. The LightCycler software automatically identifies the first maximum of the second derivative curve of the function that relates fluorescence to cycle number and this serves as the CP in the second derivative maximum method (Orta et al., 2002). This method of obtaining the CPs was used because there is no user influence in this step.

The model proposed by Pfaffl (2001) was compared to the conventionally used $\Delta\Delta C_T$ method, which assumes the same PCR efficiency for the brochocin-C and 16S rRNA genes. The Pfaffl model is a mathematical method for calculating the relative expression ratio of a target gene based on PCR efficiency and crossing point deviation (Pfaffl, 2001). Relative quantification is always based on normalization of the target gene to reference gene expression but the Pfaffl model includes further controls to standardize each reaction run with respect to RNA integrity, RT efficiency, and cDNA sample loading variations (Pfaffl, 2001). The model is dependent on the determination of real-time amplification efficiencies. The 'delta-delta method' assumes equivalent amplification efficiencies for the target and reference genes. The CPs used to calculate the relative expression ratios using the two methods are shown in Table 5.2. *C. maltaromaticum* LV17C + pGPB6 was used as the control (calibrator) strain for the 3 amplification runs with CPs of 32.18 for the brochocin-C gene and 19.91 for the 16S rRNA gene for Run 1, 32.65 and 20.00, respectively for Run 2, and 32.92 and 19.77, respectively for Run 3. There were no substantial differences between the expression ratios calculated using the two methods (Table 5.2) indicating that the PCR efficiencies of the brochocin-C and 16S RNA genes were in fact very similar. Either of the two

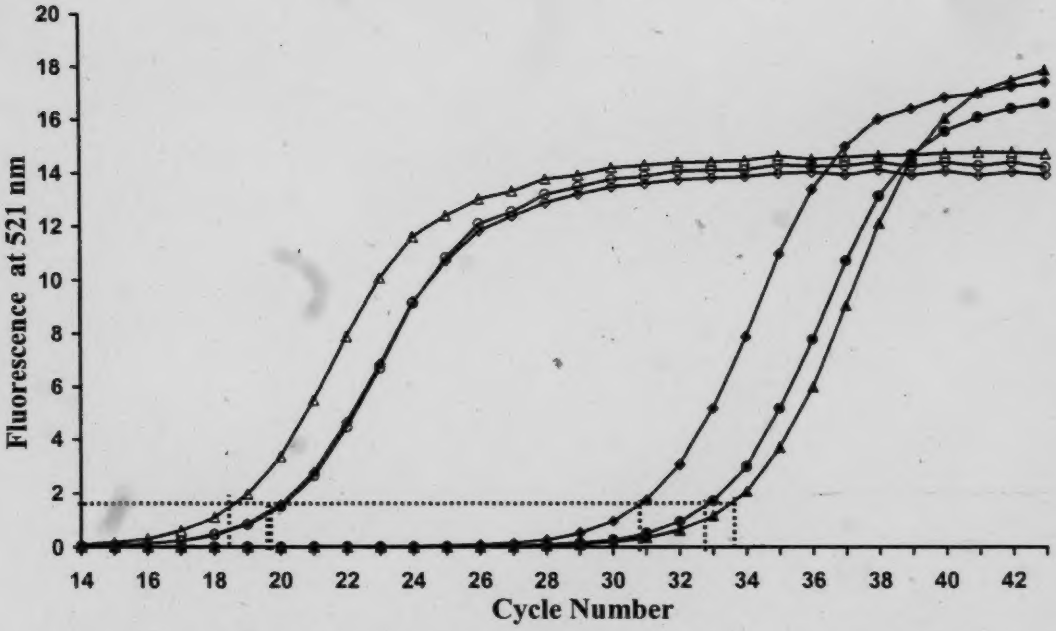

Figure 5.4. Amplification plots of brochocin-C and 16S rRNA genes using real-time PCR. Fluorescence is plotted against cycle number to obtain crossing points for calculation of relative quantification ratios. Closed symbols represent amplification of brochocin-C genes; open symbols represent amplification of 16S rRNA genes. Crossing points are shown by the intersection of the dotted lines with the amplification plots. ◆, cDNA from *C. maltaromaticum* LV17C + pGPB3; ▲, cDNA from *C. maltaromaticum* LV17C + pGPB11; ●, cDNA from *C. maltaromaticum* LV17C + pGPB6.

methods would provide reliable relative quantification ratios for the comparison of brochocin-C gene expression. Only two of the strains, *C. maltaromaticum* LV17C + pGPB2 and *C.* piscicola LV17C + pGPB3, produced expression ratios over 2.0 relative to *C. maltaromaticum* LV17C + pGPB6. The relative expression ratios for the remaining strains ranged from 0.2 to 1.6 compared to the control strain. The two strains that had relative expression ratios above 2.0 were the same two strains that were previously observed to produce higher amounts of detectable brochocin-C activity compared to the other strains (Table 5.2). It is of interest that promoter strength based on chloramphenicol resistance (Table 5.1) did not coincide with brochocin-C expression (Table 5.2) for the majority of the strains. For example, strains that exhibited resistance to high levels of chloramphenicol often had low expression ratios for brochocin-C. Thus, promoter strength based on reporter gene activity did not apply to brochocin-C gene expression. Gene expression did correlate with observed brochocin-C activity using the spot-on-lawn technique. However, it appeared that the brochocin-C genes must be expressed at a certain level before there is a detectable increase in antimicrobial activity.

This study showed that real-time PCR is a rapid, sensitive method for comparing gene expression between strains of *C. maltaromaticum* LV17C containing various promoters. Both mathematical models provided reliable information to compare brochocin-C expression between various strains of *C. maltaromaticum* LV17C; therefore, it was not necessary to develop time-consuming, labor-intensive standard curves. The relative expression ratios obtained using real-time PCR verified previously observed bacteriocin activity. This study confirmed that promoter strength based on a reporter gene such as *cat*-86 is not necessarily the same for another gene such as brochocin-C. It also appears that there is a threshold level of gene expression that needs to be reached before an increase in brochocin-C activity is detected using a biological assay that may not be very sensitive to small variations in activity. The question of whether the Sec pathway is a hurdle to brochocin-C activity is still not fully resolved even though increased activity was observed when the brochocin-C genes were under the control of two of the promoters. It is still possible that using the Sec pathway for the heterologous expression of brochocin-C, regardless of the promoter used, may result in overall decreased bacteriocin activity and future studies could investigate the use of dedicated transport mechanisms to resolve this question. The RNA isolation, reverse transcription, and real-time PCR methods used in this study could easily be applied to other bacteriocin-producing LAB to screen for promoters that enhance bacteriocin gene expression.

The majority of research on quantification of gene expression using real-time PCR has been performed using eukaryotic systems. Because of its sensitivity, speed, accuracy, and reproducibility real-time PCR has been increasingly used to quantify mRNA in very small numbers of cells and from small amounts of tissue samples from *in vivo* sources. It is being used to provide information on changes in expression levels which is aiding in the diagnosis and analysis of cancer, metabolic disorders, and autoimmune diseases (Wang et al., 1989). However, the enormous potential of real-time PCR for quantification of gene expression in prokarotic cells has recently been recognized (Corbella and Puyet, 2003; Edwards and Saunders, 2001; Fujiwara et al., 2002; Jakava-Viljanen et al., 2002; Manganelli et al., 1999; Rosato et al., 2003; Vandecasteele et al., 2001; Vaudaux et al., 2002). In this study real-time PCR was successfully used to compare the expression of brochocin-C under the control of various promoters in *C. maltaromaticum* LV17C and has potential for use in future studies to quantify and compare gene expression in other LAB.

5.4 References

Bustin, S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* **25**: 169-193.

Bustin, S.A. (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* **29**: 23-39.

Carrier, T.A., and Keasling, J.D. (1997) Controlling messenger RNA stability in bacteria: strategies for engineering gene expression. *Biotechnol Prog* **13**: 699-708.

Cheung, A.L., Eberhardt, K.J., and Fischetti, V.A. (1994) A method to isolate RNA from gram-positive bacteria and mycobacteria. *Anal Biochem* **222**: 511-514.

Corbella, M.E., and Puyet, A. (2003) Real-time reverse transcription-PCR analysis of expression of halobenzoate and salicylate catabolism-associated operons in two strains of *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **69**: 2269-2275.

Edwards, K.J., and Saunders, N.A. (2001) Real-time PCR used to measure stress-induced changes in the expression of the genes of the alginate pathway of *Pseudomonas aeruginosa*. *J Appl Microbiol* **91**: 29-37.

Fujiwara, T., Hoshino, T., Ooshima, T., and Hamada, S. (2002) Differential and quantitative analyses of mRNA expression of glucosyltransferases from *Streptococcus mutans* MT8148. *J Dent Res* **81**: 109-113.

Ginzinger, D.G. (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* **30**: 503-512.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., and Mathieu, C. (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25**: 386-401.

Goerke, C., Bayer, M.G., and Wolz, C. (2001) Quantification of bacterial transcripts during infection using competitive reverse transcription-PCR (RT-PCR) and LightCycler RT-PCR. *Clin Diagn Lab Immunol* **8**: 279-282.

Grunberg-Manago, M. (1999) Messenger RNA stability and its role in control of gene expression in bacteria and phages. *Annu Rev Genet* **33**: 193-227.

Homan, W.L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., Van Embden, J.D., and Willems, R.J. (2002) Multilocus sequence typing scheme for *Enterococcus faecium*. *J Clin Microbiol* **40**: 1963-1971.

Jakava-Viljanen, M., Avall-Jaaskelainen, S., Messner, P., Sleytr, U.B., and Palva, A. (2002) Isolation of three new surface layer protein genes (slp) from *Lactobacillus brevis* ATCC 14869 and characterization of the change in their expression under aerated and anaerobic conditions. *J Bacteriol* **184**: 6786-6795.

Johnson, M.R., Wang, K., Smith, J.B., Heslin, M.J., and Diasio, R.B. (2000) Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. *Anal Biochem* **278**: 175-184.

Jung, R., Soondrum, K., and Neumaier, M. (2000) Quantitative PCR. *Clin Chem Lab Med* **38**: 833-836.

Lekanne Deprez, R.H., Fijnvandraat, A.C., Ruijter, J.M., and Moorman, A.F. (2002) Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Anal Biochem* **307**: 63-69.

Liu, W., and Saint, D.A. (2002) Validation of a quantitative method for real time PCR kinetics. *Biochem Biophys Res Commun* **294**: 347-353.

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **25**: 402-408.

Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F.R., and Smith, I. (1999) Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol Microbiol* **31**: 715-724.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E., and Van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. *Appl Environ Microbiol* **64**: 4757-4766.

Mora, D., Fortina, M.G., Parini, C., Daffonchio, D., and Manachini, P.L. (2000) Genomic subpopulations within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationships between pediocin AcH/PA-1 producing and non-producing strains. *Microbiology* **146 (Pt 8)**: 2027-2038.

Nallapareddy, S.R., Duh, R.W., Singh, K.V., and Murray, B.E. (2002) Molecular typing of selected *Enterococcus faecalis* isolates: pilot study using multilocus sequence typing and pulsed-field gel electrophoresis. *J Clin Microbiol* **40**: 868-876.

Orta, X., Balsells, J., Olsina, J., Venereo, Y., Segovia-Silvestre, T., and Farriol, M. (2002) Quantitative analysis of ornithine decarboxylase mRNA by reverse transcription-PCR with the LightCycler system. *Clin Chem* **48**: 1779-1781.

Peirson, S.N., Butler, J.N., and Foster, R.G. (2003) Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res* **31**: e73.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Pfaffl, M.W., Horgan, G.W., and Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30**: e36.

Ramakers, C., Ruijter, J.M., Deprez, R.H., and Moorman, A.F. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339**: 62-66.

Rasmussen, R., Morrison, T., Herrmann, M., and Wittwer, C. (1998) Quantitative PCR by continuous fluorescence monitoring of a double strand DNA specific binding dye. *Biochemica* **2**: 8-11.

Ririe, K.M., Rasmussen, R.P., and Wittwer, C.T. (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* **245**: 154-160.

Rosato, A.E., Craig, W.A., and Archer, G.L. (2003) Quantitation of mecA transcription in oxacillin-resistant Staphylococcus aureus clinical isolates. *J Bacteriol* **185**: 3446-3452.

Savli, H., Karadenizli, A., Kolayli, F., Gundes, S., Ozbek, U., and Vahaboglu, H. (2003) Expression stability of six housekeeping genes: A proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol* **52**: 403-408.

Schmittgen, T.D., Zakrajsek, B.A., Mills, A.G., Gorn, V., Singer, M.J., and Reed, M.W. (2000) Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem* **285**: 194-204.

Shepard, B.D., and Gilmore, M.S. (1999) Identification of aerobically and anaerobically induced genes in *Enterococcus faecalis* by random arbitrarily primed PCR. *Appl Environ Microbiol* **65**: 1470-1476.

Sturzenbaum, S.R., and Kille, P. (2001) Control genes in quantitative molecular biological techniques: the variability of invariance. *Comp Biochem Physiol B Biochem Mol Biol* **130**: 281-289.

Talon, R., Grimont, P.A.D., Grimont, F., Gasser, F., and Boeufgras, J.M. (1988) *Brochothrix campestris* sp. nov. *Int J Syst Bacteriol* **38**: 99-102.

Vandecasteele, S.J., Peetermans, W.E., Merckx, R., and Van Eldere, J. (2001) Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during in vitro growth and under different conditions. *J Bacteriol* **183**: 7094-7101.

Vaudaux, P., Francois, P., Bisognano, C., Kelley, W.L., Lew, D.P., Schrenzel, J., Proctor, R.A., McNamara, P.J., Peters, G., and Von Eiff, C. (2002) Increased expression of clumping factor and fibronectin-binding proteins by hemB mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. *Infect Immun* **70**: 5428-5437.

Wang, A.M., Doyle, M.V., and Mark, D.F. (1989) Quantitation of mRNA by the polymerase chain reaction. *Proc Natl Acad Sci U S A* **86**: 9717-9721.

Wang, T., and Brown, M.J. (1999) mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal Biochem* **269**: 198-201.

Wilfinger, W.W., Mackey, K., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques* **22**: 474-476, 478-481.

Winer, J., Jung, C.K., Shackel, I., and Williams, P.M. (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem* **270**: 41-49.

Yin, J.L., Shackel, N.A., Zekry, A., McGuinness, P.H., Richards, C., Putten, K.V., McCaughan, G.W., Eris, J.M., and Bishop, G.A. (2001) Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. *Immunol Cell Biol* **79**: 213-221.

6. General discussion and conclusions

Although LAB have a long history of use in the fermentation of foods, the applications of LAB to improve food and health are increasingly being expanded. In recent years LAB have been investigated as food-grade delivery vehicles for vaccines (Gilbert et al., 2000; Grangette et al., 2001; Grangette et al., 2002; Langella and Le Loir, 1999; Ribeiro et al., 2002) as well as factories for the heterologous production of proteins (Arnau et al., 1997; Bernasconi et al., 2002). Numerous studies have also been conducted using LAB for the heterologous production of bacteriocins. Bacteriocins have enormous potential for use in the biopreservation of foods to prevent spoilage and enhance safety. Bacteriocins studied in our laboratory include leucocin A (Leisner et al., 1996), enterocin B (Franz et al., 1999), piscicolin 126 (Jack et al., 1996), and brochocin-C (McCormick et al., 1998). Brochocin-C is of particular interest because it has a broad antibacterial spectrum equivalent to that of nisin (Stiles, 1996) and includes spores of *Bacillus* and *Clostridium* spp. (McCormick et al., 1998).

Research in our laboratory has resulted in the heterologous expression of several bacteriocins by LAB using the general secretory pathway and plasmid vectors (Franz et al., 1999; McCormick et al., 1996; McCormick et al., 1998; McCormick et al., 1999). However, two major limitations have been noted with the strategies used to obtain heterologous production of bacteriocins: 1) plasmid instability in the absence of selective pressure; and 2) reduced detectable bacteriocin activity compared to wild-type production. If these organisms are to be used as biopreservatives both of these limitations need to be overcome. The objectives of this research aimed to address these two issues.

The first limitation addressed was the lack of plasmid stability in the absence of antibiotic pressure. To use plasmid-containing bacteria in food systems, it is essential that the plasmids that contain the bacteriocin genes remain stable for extended periods of time without selective pressure. The first part of this research focused on finding a suitable plasmid vector for the brochocin-C genes that would be stable in the absence of selective pressure. In previous studies, a rolling circle plasmid, pMG36e, was used as the vector for brochocin-C genes (McCormick et al., 1998). Because pMG36e, containing the brochocin-C construct, was not stable in the absence of erythromycin, a theta replicating plasmid available in our laboratory was investigated. A plasmid that replicated via a theta-type mechanism would likely be more stable than a rolling circle plasmid because the replication process does not result in single-stranded intermediates (Kiewiet et al., 1993). The plasmid, pTRKH2, was initially chosen because of its extensive multiple cloning site and because of its reported ability to replicate in various LAB (O'Sullivan and Klaenhammer, 1993). Plasmid, pTRKH2, had been transformed into *Lactococcus lactis*, *Enterococcus faecalis*, *Streptococcus thermophilus*, and *Lactobacillus johnsonii* (O'Sullivan and Klaenhammer, 1993) and would likely be able to replicate in *Carnobacterium* spp., the LAB chosen for this study. It was shown that pTRKH2 could replicate in *Carnobacterium* spp. and the plasmid was stable in the absence of antibiotic pressure when a construct containing the brochocin-C genes and the genetic material for export via the Sec pathway were inserted (see Chapter 2). An unexpected and unfortunate discovery was that plasmid stablility dramatically decreased when the plasmid-containing strains were incubated at low temperatures.

If LAB that heterologously produce bacteriocins are to be of use in the biopreservation of foods, it is vital that the plasmid vectors remain stable for extended periods at refrigeration temperatures. In an effort to obtain the stable production of brochocin-C at low temperatures, another plasmid was investigated. Plasmid, pCD11, an erythromycin resistant derivative of pCD3.4, is a theta-type plasmid that carries the genes for divergicin A, a bacteriocin naturally exported via the Sec pathway (Worobo et al., 1995) and replicates in *Carnobacterium* spp. The plasmid has been characterized (van Belkum and Stiles, 1999) and an appropriate restriction site was chosen for the insertion of the brochocin-C construct (see Chapter 2). This brochocin-C containing plasmid, pCDB, was extremely stable in *Carnbacterium* spp. during incubation at 4 and 25°C. Thus the first objective of this thesis was met with the production of brochocin-C using a plasmid that was stable in the absence of antibiotic pressure during extended incubation in broth media at both ambient and refrigeration temperatures. Future studies need to be conducted to determine if plasmid stability is affected when *Carnobacterium* strains are added to food matrices and stored for extended periods under refrigeration conditions. It also remains to be determined if food components have an effect on brochocin-C activity.

Plasmid, pCD3.4, is promising as a food-grade vector since it was originally isolated from *C. divergens* NCIMB 702855, an organism associated with foods, and does not contain genes encoding antibiotic resistance. This plasmid is ideal for the heterologous production of bacteriocins used for the biopreservation of foods because of its excellent stability at both ambient and refrigeration temperatures. Other researchers have achieved similar success using small theta-replicating plasmids for heterologous production. Mesentericin Y105 has been heterologously produced by *Leuconostoc cremoris* at a level similar to that of the wild-type producer using pTXL1, a small cryptic plasmid that was developed into a food-grade vector (Biet et al., 2002). The plasmid was also very stable in *Lc. mesenteroides* subsp. *dextranicum* during extended growth at 30°C under nonselective conditions with 100% of the clones containing the plasmid after 100 generations (Biet et al., 2002). However, the effect of low temperature on plasmid stability was not reported. Low temperature was observed to cause a rapid loss of pTRKH2 from *Carnobacterium* spp. but not affect the stability of pCD11, both theta-type plasmids. The influence of low temperature on theta-replicating plasmids had not previously been reported in the literature and needed further investigation if plasmids were to be effectively used as vectors for bacteriocin genes. The stability of five theta-type plasmids transformed into *C. maltaromaticum* UAL26 was determined during incubation at 4 and 25°C (see Chapter 3). The plasmids that were investigated were chosen based on their ability to replicate in several species of LAB and on their reported segregational stability in LAB. The ability of the original host strains from which these plasmids were derived were observed for their ability to grow in broth incubated at 2 and 4°C. It was hypothesized that the growth temperature characteristics of the original host strain could reflect the plasmid stability at that temperature. For example, if the host strain could grow at low temperatures then derivatives of the plasmid isolated from that strain would be stable during extended storage at low temperature. If the host strain could not grow at refrigeration temperatures then its derivative plasmids would not be stable at low temperature. This in fact appeared to be the case for four of the plasmids that we obtained from their corresponding host organisms. For one of the derivative plasmids, pHW800, the host strain could not be obtained and therefore its growth temperature characteristics could not be determined. The results of this study indicate that knowing whether the original host strain has the ability to grow at refrigeration temperatures may aid in selecting plasmids that have a greater likelihood of remaining stable in a heterologous host stored at low temperatures. To our knowledge the influence of low temperature on plasmid stability has not been previously reported.

Researchers have shown that the replication region is involved in plasmid stability at high temperatures (Duan et al., 1999; Frere et al., 1998; Horng et al., 1991). It remained to be determined whether the replication region is also involved in plasmid stability at low temperatures. Because pCD11, a derivative of pCD3.4, demonstrated excellent stability in *C. maltaromaticum* UAL26 during extended incubation at 4°C and because pCD3.4 shows promise as a food-grade vector, its replication region was investigated for effect on stability. A fragment containing 12 and 22 base pairs repeats, putative DnaA box and origin of replication, and the *repA* gene was inserted into the multiple cloning site of pTRKH2, a plasmid that was not stable at 4°C. A shortened version that lacked the DNA iterons and the *repA* gene but contained a region reported to affect segregational stability of pCD3.4 (van Belkum and Stiles, 1999) was also inserted into pTRKH2. The longer fragment enhanced the stability of pTRKH2 dramatically during incubation at 4 and 25°C whereas the shortened version resulted in a slight decline in plasmid stability at 25°C and did not improve stability at 4°C (see Chapter 3). Therefore, the replication region, including the *repA* gene and DNA iterons, was important for stability at low temperatures. In order to more fully characterize the regions influencing stability at low temperatures, experiments using deletion derivatives should be conducted. It would also be of interest to investigate the replication regions of other stable plasmids such as pCaT and pHW800 to determine whether they also can enhance the stability of a non-stable plasmid during incubation at low temperature. These types of studies were beyond the scope of this thesis but additional information gained from investigations into the influence of low temperature on plasmid stability and replication would be valuable.

Brochocin-C was heterologously produced by *Carnobacterium* spp. using a plasmid that was stable in the absence of antibiotic pressure but was also stable during extended storage at low temperature. However, the second major limitation associated with the heterologous production of brochocin-C utilizing the Sec pathway and a plasmid vector was the reduction of bacteriocin activity compared to production by the wild-type organism. In an attempt to enhance brochocin-C production, several promoters of varying strengths were investigated (see Chapter 4). Other researchers have successfully used constitutive promoters from LAB to increase the expression of heterologous genes (Kahala and Palva, 1999; Leenhouts et al., 1998; Piard et al., 1997). To investigate the effect of various promoters on brochocin-C activity, promoters were isolated and screened for activity in *C. maltaromaticum* LV17C using the promoter-screening vector, pGKV210 (van der Vossen et al., 1985), a plasmid that replicated in carnobacteria. Initially four other lactococcal promoters in addition to P32 were investigated for strength in *C. maltaromaticum* LV17C and for their effect on brochocin-C activity. These constitutive promoters have been used for the expression of various heterologous proteins with much success. Depending on the heterologous protein and host organism, the strength of the lactococcal promoters varied. For example, P59 was reported to be the strongest promoter controlling the expression of the *cat*-86 gene in *Lactococcus lactis* IL1403 but was observed to be the weakest promoter in *C. maltaromaticum* LV17C (see Chapter 4). Likewise, chitinase activity was increased to a greater extent when the gene was under the control of P59 compared to P32 (Brurberg et al., 1994) and the *emm6* gene was expressed at a higher level using P59 compared to P23 (Piard et al., 1997). However, P59 yielded significantly lower endolysin activity compared to P32 (Gaeng et al., 2000). In all of these cases, the heterologous host was *L. lactis*. Using these promoters in this study did not have any effect on brochocin-C activity and therefore even though these lactococcal promoters demonstrated varying degrees of strength based on a reporter gene, they apparently had no effect on brochocin-C expression (see Chapter 4). A similar strategy, using a reporter gene, employed by van der Vossen et al. (1987)

was utilized to isolate and screen native promoters from *C. maltaromaticum* LV17C and *C. maltaromaticum* UAL26. These native promoters demonstrated a wide variety of strengths based on resistance of *C. maltaromaticum* LV17C to different concentrations of chloramphenicol. However, the majority of these promoters also did not have any apparent effect on brochocin-C expression. Only two of the strains produced twice the amount of brochocin-C activity compared to the remaining strains. It was possible that the various promoters were influencing the level of brochocin-C gene expression but other limitations such as translational efficiency or export via the Sec pathway were limiting brochocin-C detection. To determine if the promoters were affecting brochocin-C transcript levels, the expression of the brochocin-C genes under the control of the various promoters was analyzed.

Comparative quantification of brochocin-C gene expression was performed using real-time PCR. This technology allows for the sensitive, rapid, and accurate comparison of gene expression. Real-time PCR measures the number of cycles necessary to detect a fluorescent signal and the resulting threshold cycle is used to determine the starting level of a particular mRNA (Lekanne Deprez et al., 2002). The basis of quantification using real-time PCR is that the amount of amplified target is directly proportional to the input amount of target during the exponential range of amplification (Schmittgen et al., 2000). Comparative relative quantification was used in this study because this method would provide sufficient information on the effect of promoter activity on brochocin-C expression (see Chapter 5). This method is much simpler, less labor-intensive, and less costly compared to absolute quantification using standard curves. For the purpose of this study it was not necessary to determine the exact copy number of the brochocin-C genes in each strain. One of the challenges associated with relative quantification is finding a good reference gene to use for normalization of results. The reliability of relative quantification largely depends on the normalization of unwanted variations between samples and constantly expressed genes such as housekeeping genes are used as internal controls (Giulietti et al., 2001; Pfaffl et al., 2002; Savli et al., 2003). Because of the lack of published information on housekeeping genes in *Carnobacterium* spp., the 16S rRNA gene was selected. Fortunately, the sequence of the 16S rRNA gene is highly conserved amongst LAB and this gene proved to be a very good reference gene (see Chapter 5). Using mathematical models, the expression of the brochocin-C gene under the control of the various promoters was compared. The findings of this study confirmed the previously observed results. Only two of the nineteen strains produced higher brochocin-C activity when the brochocin-C construct was under the control of two native promoters from *C. maltaromaticum* LV17C (see Chapter 4). These two promoters exhibited different strengths based on chloramphenicol resistance. However, when the brochocin-C construct was under their control, the same amount of brochocin-C activity was detected. Relative comparison using real-time PCR showed that brochocin-C gene expression from these two strains was also higher than the other strains when compared to a calibrator strain. The relative expression ratios for these two strains were greater than 2.0 whereas all of the remaining strains had relative expression ratios less than 2.0 (see Chapter 5). Using real-time PCR to compare gene expression verified that the majority of promoters analyzed did not increase brochocin-C expression. The results of these studies confirmed that promoter strength is context dependent (Solem and Jensen, 2002) and promoter strength based on reporter genes is not necessarily the same for target genes. This information can be applied to search for other promoters that may increase bacteriocin expression. Future studies could include the development of a synthetic library of promoters that specifically increase the expression of particular bacteriocins in heterologous LAB such as *Carnobacterium* spp. The generation of a synthetic promoter library has already proven to successful for enhancing gene expression in *L. lactis* and *E. coli* (Jensen and Hammer, 1998). Numerous promoters could be screened for strength based on expression of a bacteriocin gene rather than a reporter gene using real-time PCR. Real-time technology is relatively fast, simple, and reliable and is rapidly becoming the method of choice for gene expression studies.

It was determined that promoters did have an effect on brochocin-C gene transcript levels and it appears that a threshold level needs to be reached before an increase in brochocin-C activity is detected. However, it remains to be determined whether the Sec pathway could also have an effect on the amount of brochocin-C activity being detected. Future studies investigating the use of the Sec pathway for export of bacteriocins and an alternative approach of using dedicated transport mechanisms would be valuable. It would also be of interest to determine the effect of the promoters used in this research on the gene expression of other bacteriocins.

Overall, the objectives of this thesis have been met. New information on plasmid stability, promoter activity, and brochocin-C gene expression has been generated and can be applied in future research to enhance the heterologous production of bacteriocins by LAB.

6.1 References

Arnau, J., Hjerl-Hansen, E., and Israelsen, H. (1997) Heterologous gene expression of bovine plasmin in *Lactococcus lactis*. *Appl Microbiol Biotechnol* **48**: 331-338.

Bernasconi, E., Germond, J.E., Delley, M., Fritsche, R., and Corthesy, B. (2002) *Lactobacillus bulgaricus* proteinase expressed in *Lactococcus lactis* is a powerful carrier for cell wall-associated and secreted bovine beta-lactoglobulin fusion proteins. *Appl Environ Microbiol* **68**: 2917-2923.

Biet, F., Cenatiempo, Y., and Fremaux, C. (2002) Identification of a replicon from pTXL1, a small cryptic plasmid from *Leuconostoc mesenteroides* subsp. *mesenteroides* Y110, and development of a food-grade vector. *Appl Environ Microbiol* **68**: 6451-6456.

Brurberg, M.B., Haandrikman, A.J., Leenhouts, K.J., Venema, G., and Nes, I.F. (1994) Expression of a chitinase gene from *Serratia marcescens* in *Lactococcus lactis* and *Lactobacillus plantarum*. *Appl Microbiol Biotechnol* **42**: 108-115.

Duan, K., Liu, C.Q., Liu, Y.J., Ren, J., and Dunn, N.W. (1999) Nucleotide sequence and thermostability of pND324, a 3.6-kb plasmid from *Lactococcus lactis*. *Appl Microbiol Biotechnol* **53**: 36-42.

Franz, C.M., Worobo, R.W., Quadri, L.E., Schillinger, U., Holzapfel, W.H., Vederas, J.C., and Stiles, M.E. (1999) Atypical genetic locus associated with constitutive production of enterocin B by *Enterococcus faecium* BFE 900. *Appl Environ Microbiol* **65**: 2170-2178.

Frere, J., Benachour, A., Giard, J.C., Laplace, J.M., Flahaut, S., and Auffray, Y. (1998) A new theta-type thermosensitive replicon from *Lactococcus lactis* as an integration vector for *Enterococcus faecalis*. *FEMS Microbiol Lett* **161**: 107-114.

Gaeng, S., Scherer, S., Neve, H., and Loessner, M.J. (2000) Gene cloning and expression and secretion of *Listeria monocytogenes* bacteriophage-lytic enzymes in *Lactococcus lactis*. *Appl Environ Microbiol* **66**: 2951-2958.

Gilbert, C., Robinson, K., Le Page, R.W., and Wells, J.M. (2000) Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in *Lactococcus lactis*. *Infect Immun* **68**: 3251-3260.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., and Mathieu, C. (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25**: 386-401.

Grangette, C., Muller-Alouf, H., Goudercourt, D., Geoffroy, M.C., Turneer, M., and Mercenier, A. (2001) Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant *Lactobacillus plantarum*. *Infect Immun* **69**: 1547-1553.

Grangette, C., Muller-Alouf, H., Geoffroy, M., Goudercourt, D., Turneer, M., and Mercenier, A. (2002) Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and in vivo persistence. *Vaccine* **20**: 3304-3309.

Horng, J.S., Polzin, K.M., and McKay, L.L. (1991) Replication and temperature-sensitive maintenance functions of lactose plasmid pSK11L from *Lactococcus lactis* subsp. *cremoris*. *J Bacteriol* **173**: 7573-7581.

Jack, R.W., Wan, J., Gordon, J., Harmark, K., Davidson, B.E., Hillier, A.J., Wettenhall, R.E., Hickey, M.W., and Coventry, M.J. (1996) Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. *Appl Environ Microbiol* **62**: 2897-2903.

Jensen, P.R., and Hammer, K. (1998) The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. *Appl Environ Microbiol* **64**: 82-87.

Kahala, M., and Palva, A. (1999) The expression signals of the *Lactobacillus brevis slpA* gene direct efficient heterologous protein production in lactic acid bacteria. *Appl Microbiol Biotechnol* **51**: 71-78.

Kiewiet, R., Kok, J., Seegers, J., Venema, G., and Bron, S. (1993) The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **59**: 358-364.

Langella, P., and Le Loir, Y. (1999) Heterologous protein secretion in *Lactococcus lactis*: a novel antigen delivery system. *Braz J Med Biol Res* **32**: 191-198.

Leenhouts, K., Bolhuis, A., Boot, J., Deutz, I., Toonen, M., Venema, G., Kok, J., and Ledeboer, A. (1998) Cloning, expression, and chromosomal stabilization of the *Propionibacterium shermanii* proline iminopeptidase gene (*pip*) for food-grade application in *Lactococcus lactis*. *Appl Environ Microbiol* **64**: 4736-4742.

Leisner, J.J., Greer, G.G., and Stiles, M.E. (1996) Control of beef spoilage by a sulfide-producing *Lactobacillus sake* strain with bacteriocinogenic *Leuconostoc gelidum* UAL187 during anaerobic storage at 2 degrees C. *Appl Environ Microbiol* **62**: 2610-2614.

Lekanne Deprez, R.H., Fijnvandraat, A.C., Ruijter, J.M., and Moorman, A.F. (2002) Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Anal Biochem* **307**: 63-69.

McCormick, J.K., Worobo, R.W., and Stiles, M.E. (1996) Expression of the antimicrobial peptide carnobacteriocin B2 by a signal peptide-dependent general secretory pathway. *Appl Environ Microbiol* **62**: 4095-4099.

McCormick, J.K., Poon, A., Sailer, M., Gao, Y., Roy, K.L., McMullen, L.M., Vederas, J.C., Stiles, M.E., and Van Belkum, M.J. (1998) Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. *Appl Environ Microbiol* **64**: 4757-4766.

McCormick, J.K., Klaenhammer, T.R., and Stiles, M.E. (1999) Colicin V can be produced by lactic acid bacteria. *Lett Appl Microbiol* **29**: 37-41.

O'Sullivan, D.J., and Klaenhammer, T.R. (1993) High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene* **137**: 227-231.

Pfaffl, M.W., Horgan, G.W., and Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30**: e36.

Piard, J.C., Hautefort, I., Fischetti, V.A., Ehrlich, S.D., Fons, M., and Gruss, A. (1997) Cell wall anchoring of the *Streptococcus pyogenes* M6 protein in various lactic acid bacteria. *J Bacteriol* **179**: 3068-3072.

Ribeiro, L.A., Azevedo, V., Le Loir, Y., Oliveira, S.C., Dieye, Y., Piard, J.C., Gruss, A., and Langella, P. (2002) Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microbiol* **68**: 910-916.

Savli, H., Karadenizli, A., Kolayli, F., Gundes, S., Ozbek, U., and Vahaboglu, H. (2003) Expression stability of six housekeeping genes: A proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol* **52**: 403-408.

Schmittgen, T.D., Zakrajsek, B.A., Mills, A.G., Gorn, V., Singer, M.J., and Reed, M.W. (2000) Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem* **285**: 194-204.

Solem, C., and Jensen, P.R. (2002) Modulation of gene expression made easy. *Appl Environ Microbiol* **68**: 2397-2403.

Stiles, M.E. (1996) Biopreservation by lactic acid bacteria. *Antonie Van Leeuwenhoek* **70**: 331-345.

van Belkum, M.J., and Stiles, M.E. (1999) Characterization of a theta-type plasmid from *Carnobacterium divergens* and its use as a bacteriocin expression vector. In *Sixth Symposium on Lactic Acid Bacteria: Genetics, Metabolism and Applications* Veldhoven, the Netherlands: Federation of European Microbiological Societies and Netherlands Society for Microbiology.

van der Vossen, J.M., Kok, J., and Venema, G. (1985) Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. *Appl Environ Microbiol* **50**: 540-542.

van der Vossen, J.M., van der Lelie, D., and Venema, G. (1987) Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. *Appl Environ Microbiol* **53**: 2452-2457.

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C., and Stiles, M.E. (1995) A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. *J Bacteriol* **177**: 3143-3149.